Research Article

Orbitofrontal neurons acquire responses to ‘valueless’ Pavlovian cues during unblocking

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Abstract
The orbitofrontal cortex (OFC) has been described as signaling outcome expectancies or value. Evidence for the latter comes from the studies showing that neural signals in the OFC correlate with value across features. Yet features can co-vary with value, and individual units may participate in multiple ensembles coding different features. Here we used unblocking to test whether OFC neurons would respond to a predictive cue signaling a ‘valueless’ change in outcome flavor. Neurons were recorded as the rats learned about cues that signaled either an increase in reward number or a valueless change in flavor. We found that OFC neurons acquired responses to both predictive cues. This activity exceeded that exhibited to a ‘blocked’ cue and was correlated with activity to the actual outcome. These results show that OFC neurons fire to cues with no value independent of what can be inferred through features of the predicted outcome.
Introduction

The orbitofrontal cortex (OFC) is often described as signaling either an outcome expectancy, implying a knowledge of the features of the impending outcome (1-5), or a value that exists independent of those features (6, 7). Support for such pure or abstract value encoding comes largely from reports that single unit activity and the blood-oxygen level dependent (BOLD) response in the OFC tracks value, independent of outcome features such as identity or location or even the response required to obtain the outcome (8-10).

Yet in all these studies, outcome features could still be the underlying basis of the neural signals. This is because a signal that varies with outcome value might be encoding features that co-vary with value. This is true even if the signal correlates with value across different outcomes, since some features will be common across the limited number of outcomes used in any particular experiment. For example, a common neural code that seems to be similar for two juices may track their sweetness or another dimension such as number or size that increases with value. Further, any neural element (voxel or single unit) may participate in ensembles responding to more than one feature, so it is also possible that a particular element that appears not to distinguish specific features of different outcomes is in fact coding independent features that co-vary with each outcome’s value.

So how can we address whether the OFC signals features of impending outcomes versus value independent of those features? One way is to strip away or “block” the value portion of the outcome during learning, while leaving unblocked – free to enter into associations – the outcome’s unique sensory and other features. This can be done by pairing a “target” cue with a rewarding outcome in the presence of a cue that has been previously trained to predict a differently-flavored, but similarly-valued outcome. When this is done, the previously conditioned cue predicts the general value that is common to the two outcomes, but does not predict the unique features that distinguish the new outcome (note features are not limited to sensory properties, but might include the outcome
timing, location, temperature, size, number, etc). As a result, the target cue acquires associations
with the unique features of the new outcome but not with its general or common currency value (11,
12). If OFC neurons represent only a general or common currency value, divorced from features,
then they should respond no more to such a target cue than to a completely blocked cue (13).
However, if OFC neurons represent outcome features, independent of value, then they should
respond to the target cue just as they do to a cue that has been explicitly unblocked by increasing
the amount of the outcome delivered. Indeed, both pure value and outcome expectancy accounts of
OFC function would predict neural activity to such an unblocked cue, but only an outcome
expectancy account predicts encoding of the target cue signaling a valueless change in outcome
flavor.

Results

We recorded single-unit activity in the OFC in six rats during an odor-based unblocking task (Fig.
1A). Prior to implantation with electrodes rats were trained to sample an odor in a central port
following house light illumination and then respond to a reward well below for two drops of Nestlé’s
flavored milk (chocolate or vanilla, counterbalanced). This training was meant to establish the initial
odor as a reliable predictor of a specific flavor and number of drops of milk. Each rat had extensive
experience with both flavors, thus neither flavor was novel. Following initial training rats were
implanted with microelectrodes in the OFC. When recovered from surgery, rats were retrained on
the initial odor; after retraining, each rat underwent 7-9 rounds of unblocking.

Each round of unblocking began with two days of training and consisted of four trial types. One type
was a reminder; the initially trained odor was followed by the expected outcome. On the other three
trial types (blocked, number, flavor), rats were presented with the initially trained odor, followed
immediately by one of three novel odors. On “blocked” trials, the novel odor was followed by the
expected two drops of the same flavor used in initial training. This outcome is fully predicted by the
initial odor, thus the novel odor should be blocked from acquiring associative significance (13). On
“flavor” trials, the novel odor was followed by two drops of the flavor not used in initial training (i.e. chocolate or vanilla). Here the value is unchanged, since the two flavors are equally preferred and the same amount is delivered, but the features of the outcome are different. Thus the novel target odor should enter into associations with the unique features of the outcome (11, 12). On “number” trials, the novel odor was followed by an additional drop of the flavor used in the initial training. Since the initial odor does not predict anything after the second drop, the novel odor should enter into associations with both the features and the additional value of the outcome presented in the third drop (14).

To ensure that learning in the flavor condition did not result from an explicit shift in value, two types of preference tests were administered in conjunction with the unblocking training procedures. In one test, given on days separate from unblocking, preference for each flavor over water was assessed. Both flavors were highly preferred to water (Fig. 1B), demonstrating both are highly palatable. The more important preference test came just following unblocking sessions, from which the critical neural data came. In these tests, the two milk flavors were pitted directly against one another. This test is critical to demonstrate that specific satiety to one flavor did not develop over the course of the unblocking session, a finding that would strongly suggest different valuation of the two flavors. Indeed, we found no evidence of a preference in these tests (Fig. 1C). In both types of tests the locations of the bottles were swapped every 20-30 seconds, meaning that rats were required to switch locations if the solution did in fact differ in value. This pattern was present when either milk flavor was compared to water but was absent when the two flavors were directly compared. The consumption data demonstrate that both flavors were highly palatable yet of equivalent value. Finally, to ensure the two flavors were discriminable we subjected another set of rats to a selective conditioned flavor aversion procedure. After initial exposure to both milk flavors, consumption of one flavor (fully counterbalanced) was devalued by pairing with LiCl-induced nausea while consumption of the other was paired with saline injection that is of minimal consequence. At no point did rats show a preference for the chocolate or vanilla flavor but all rats selectively reduced consumption of...
the devalued flavor. This was apparent both in conditioning (Fig. 1/Supp. 1) and in the final choice test (Fig 1D). Thus extensive consumption testing indicated that the flavors used were of equivalent value but readily discriminable.

In the unblocking sessions rats were sensitive to presentation of the novel odors, exhibiting longer latencies to respond at the reward well following odor sampling on these three trial types. Longer latencies to the novel odors were most apparent on the very first trial of each session, particularly on day 1. In support, ANOVA revealed a main effect of trial (F₁,₄₇²>2, p's<0.01) and a trial x day interaction (F₁,₄₇²=34.73, p<0.01). However the rats also learned that the two novel odors that predicted changes in the outcome were meaningful. This was evident in the extinction probe test in which they initially spent more time in the fluid well following sampling of the flavor and number odors than following the blocked odor (Fig. 1E).

We recorded 240 single units during the first day of unblocking and 220 units on the second unblocking day in 48 rounds of training across all 6 rats (Fig. 1F). To address our hypothesis, units from both unblocking days were screened for phasic responses to one of the four odors using a t-test, which compared firing rates during the ITI and novel odor period (significance level = p<0.0125; Bonferroni correction). This screen found 135 units (Day 1=79, Day 2=56) that showed a significant increase in firing to at least one of the odor cues. The majority - 98/135 or 73% of the neurons within this population - exhibited activity that fell into one of two categories (see Fig. 2/Supp. 1 for analysis of other neurons). We will consider each category in turn below.

The first major category, not directly anticipated by our hypothesis, consisted of neurons (55/135, Fig. 2A) that showed a significant phasic response to each of the four odor cues. Since these neurons fired to all of the odors, even the blocked odor, their firing cannot be easily explained as signaling information about the predicted outcomes. However they might be signaling information about the cues themselves, such as their shared sensory features or intrinsic salience. Unlike shared sensory features, salience should be higher for the novel cues than for the pre-trained, initial
odor in our design, and this pattern should be most noticeable early in training when the novel cues were first presented. As a population, these neurons did show greater activity to the blocked, number and flavor odors than to the more familiar, initial odor (Fig. 2B). This effect was present despite the fact that we did not select based on this criterion. Further analyses of the individual units showed that many exhibited significantly higher firing to the novel odors than to the initial odor (Fig. 2C), and few exhibited differences in firing among the 3 novel odors (Fig. 2D, E). Moreover, when we examined the firing of neurons in this population on the first 10 trials of unblocking (31/79 odor-responsive neurons; Fig. 2/Supp. 2), analyzing the difference in firing between the novel and initial odor cues in a sliding, 300-ms window across each trial, we found that activity in this population was maximal at the onset of the novel odors and on the first exposure and then declined rapidly on subsequent trials (Fig. 2F). This same pattern held when each novel odor was analyzed separately (Fig. 2/Supp. 3). Further, this pattern was only seen on the first day of unblocking (Fig. 2/Supp. 4). This pattern of activity is consistent with signaling of the salience of these cues.

The second major category, of greater relevance to our hypothesis, consisted of neurons (43/135, Fig. 3A-C) that showed a significant phasic response the flavor and/or number odors (but did not fire to all four odors). Activity across this population was greater in response to the two predictive odor cues than to either the blocked or initial odors (Fig. 3D), and an analysis of individual units showed that nearly all of these neurons (38/43) fired more to the predictive odors than to the blocked one (Fig. 3E). This result marks these neurons as candidates for encoding of associative information or meaning, since this is the primary feature that distinguishes the two odor cues from the blocked odor.

Interestingly, these neurons did not appear to distinguish, at least as a population, between the flavor and number odors. For example, they showed similar levels of activity in response to both the flavor and the number odor (Fig. 3D and 3F), and when we examined the firing of neurons in this population on the first 10 trials of unblocking (25/79 odor-responsive neurons, Fig. 3/Supp.), we
found that differential firing to each cue developed at a similar rate during training (Fig. 3G-J). The acquisition of differential firing to the flavor and number odors demonstrates that selective odor encoding was not driven by physical properties of the odors. If neurons were encoding the odor itself, independent of its outcome signaling, this would have been apparent on the very first trial. Thus, as a population, these neurons responded more strongly to the unblocked ‘flavor’ and ‘number’ odors than to the ‘blocked’ odor. Even more striking, the population responded similarly to a cue signaling a valueless change in the outcome flavor as they did to a cue that signaling that more of the outcome would be delivered.

Similar numbers of neurons fired to the flavor and number cues (flavor: 31, number: 37; $\chi^2=0.3$, $p=0.47$). In our design, firing to the flavor cue cannot be readily explained as signaling general or common value. Thus these data confirm that many OFC neurons signal associative meaning independent of at least a general value. In support of this, odor firing in the flavor population, as well as the number population, was positively correlated with firing to the actual outcome delivered on each of these trials (Fig 4). This relationship supports the idea that cue-evoked activity in the flavor population is signaling features of the new outcome.

Discussion

Neural signals in the OFC are often described as representing either outcome expectancies or abstract value. Although many studies have argued for one or the other, few have used behavioral designs that clearly dissociate predictions of these two hypotheses. Here we tried to address this question by using an unblocking procedure to strip away or “block” the abstract value of the outcome during learning, while leaving unblocked – free to enter into associations – the outcome’s sensory and other unique features. This approach revealed two distinct populations of OFC neurons.
One population consisted of neurons that fired immediately on initial presentation of all three target cues, perhaps reflecting these cues’ novelty or salience. While unexpected and not directly relevant to the question motivating this study, this finding is consistent with reports of neural correlates of salience in OFC (15, 16) and with studies implicating the OFC in phenomena such as latent inhibition, set formation, and even auto-shaping (17-19) which depend in part on the appropriate attribution of salience to cues. Together these results point to a largely unappreciated role for this area in the modulation of attention for the purposes of learning (20). Of course novelty is just one instance of ‘salience’. Modern learning theories describe salience as a function of both intrinsic and acquired properties. Within this framework, this population was correlated with intrinsic salience.

The second population, of more direct relevance to our hypothesis, consisted of neurons that fired preferentially to the target cues that predicted changes in the outcome flavor. Nearly all of these neurons fired more to these cues than to the similarly trained but fully blocked cue, and this activity was acquired with learning, a pattern consistent with signaling of the associative significance or meaning of these cues (or possibly their acquired salience). Importantly, the acquired neural activity was observed to both the explicitly unblocked “number” cue as well as to the “flavor” cue, which was unblocked by shifting the features of the expected outcome while holding the value constant. This was accomplished by using two differently-flavored but similarly-preferred outcomes (Fig 1c-d). The lack of any flavor preference makes it unlikely that the cue added prior to this manipulation acquired what might be termed a general or cached value. Indeed, in prior work we have found that responding to this target cue in the probe test is dissociable from even the smallest animal-by-animal differences in conditioned or unconditioned responding to the two outcomes used (21), indicating that it is not driven by any sort of shift in value that might accrue to the cue.

Instead conditioned responding to a target cue unblocked by shifting the identity of the outcome seems to be particularly dependent upon the unexpected outcome’s unique features, at least in comparison to an explicitly unblocked cue. As evidence of this, it has been shown that cues trained
in this manner support behavior that is more sensitive to (in fact completely dependent upon) the
features of the predicted outcome – or value inferred through those features – than similar behaviors
supported by cues directly paired with reward in isolation. For example, conditioned reinforcement
supported by a normally trained cue is insensitive to devaluation of the predicted outcome (22);
however if the cue is trained like the "flavor" cue in the current experiment, then devaluation of the
predicted outcome completely abolishes the ability of the cue to serve as a conditioned reinforcer
(11). This result indicates that a cue trained in this manner has little or no intrinsic, cached or
acquired value except what can be inferred through knowledge of the features of the outcome. This
cue's special link to the sensory features of the outcome is also apparent in that such cues retain the
ability to support Pavlovian-to-instrumental transfer when that transfer is specific to the outcome
(12). Interestingly outcome-specific transfer is both insensitive to devaluation (23) and disrupted by
OFC lesions (4), results which are difficult to reconcile with the view that the OFC is directly involved
in the representation of value.

That OFC neurons developed robust responses to such a valueless cue indicates that many OFC
neurons – more than half of the population responsive to the acquired significance of the cues –
represent associative features that must be, strictly speaking, independent of general or common
value. Notably this population included neurons that fired only to the flavor cue as well as neurons
that participated in both the flavor and number ensembles. Such dual encoding would be expected
if, as suggested earlier, individual neurons are not labeled lines but participate in ensembles coding
more than one outcome feature (flavor, number, temperature, location, timing, etc.).

Of course some neurons fired preferentially to the valued, number cue. The firing of these neurons
could reflect the general value that accrued to this cue during training. However such firing could
equally well reflect associations with features of the additional outcome delivered on these trials,
known to develop when additional rewards are delivered during unblocking (24). While it is
impossible to say for sure, the similarities in the overall level of neural activity to the flavor and
number cues, their similar rates of development with learning, and the finding that neurons do
develop activity to a valueless cue make the latter explanation the most parsimonious.

If OFC neurons signal associations between cues and specific outcome features, this would accord
well with results showing that the OFC is not necessary when behavior – or learning – can be
accomplished using general value alone. For example, the OFC is not required for simple Pavlovian
or instrumental conditioning (4, 25-27) discrimination learning (27-30), extinction by reward omission
(31), transfer (4), and even perhaps reversal learning (32), all of which can be accomplished without
reference to specific information about predicted outcomes. Similarly both blocking and unblocking
– when it can be accounted for by value – do not require the OFC (11, 33). While the preserved
function in these studies could reflect compensation by other areas, it must at least call into question
the idea that OFC, writ large, is fundamental to all behavior that reflects value, instead highlighting
suggestions that common value representation may at least be limited to the medial subregion (34,
35). Indeed recent work in humans has shown that OFC represents specific outcome features and
that more lateral orbital areas represent those outcomes in a way that is dependent upon prior cues
(36). Thus far from signaling general value about outcomes without regard to their features and
attendant events, this work shows that the OFC maintains highly specific representations.

Such highly specific representations are consistent with observations that the OFC is necessary for
superficially similar behaviors (Pavlovian or instrumental responding, discriminations, even learning)
when they require knowledge of the outcome features in order to recognize errors or to derive or
infer a value (4, 25-27, 33). This is even true in the current paradigm, where we have shown that the
OFC is required for the development of conditioned responding to the target cue paired with a shift
in outcome identity but not to the target cue paired with additional outcome (33). Our present finding
of robust encoding of a valueless Pavlovian cue that exceeds that of a blocked cue, and is
equivalent to a cue paired with additional outcome, provides further support for outcome expectancy
theories of OFC function.
Materials and Methods

Subjects: Male Long-Evans rats were obtained at 200-250g from Charles River Labs, (Wilmington, MA). Rats were tested at the University of Maryland School of Medicine and the NIDA-IRP in accordance with SOM and NIH guidelines (12-CNRB-108).

Surgery and Histology: Using aseptic, stereotaxic surgical techniques, a drivable bundle of sixteen, 25 µm diameter FeNiCr wires (Stablohm 675, California Fine Wire, Grover Beach, CA) was chronically implanted dorsal to OFC in the left hemisphere at 3.0 mm anterior to bregma, 3.2 mm laterally, and 4.0 mm ventral to the surface of the brain in each rat. Immediately prior to implantation, these wires were freshly cut with surgical scissors to extend ~1 mm beyond the cannula and electroplated with platinum (H2PtCl6, Aldrich, Milwaukee, WI) to an impedance of ~300 kΩ. At the end of the study, the final electrode position was marked by passing a 15 µA current through each electrode. The rats were then perfused, and their brains removed and processed for histology using standard techniques.

Blocking Task: Recording was conducted in grounded aluminum chambers approximately 18” on each side with sloping walls narrowing to an area of 12” x 12” at the bottom. A central odor port was located above a fluid well on a panel in the right wall of each chamber. Two lights were located above the panel. The odor port was connected to an airflow dilution olfactometer to allow the rapid delivery of olfactory cues to the odor port; odors where chosen from compounds obtained from International Flavors and Fragrances (New York, NY). The fluid well was connected to lines controlling the independent delivery of the fluid rewards. Task control was implemented via computer running a behavioral program written in C++.

Prior to implantation with microelectrodes, rats were water deprived by restricting daily access to 1 hr following each training session. Water-deprived rats were progressively shaped to hold in the
odor port for 1s to receive two drops of water at the well. After shaping, rats received further training
until they were proficiently responding for the initial odor in order to receive two boli of milk (vanilla or
chocolate-flavored, counterbalanced); this involved as many as 15 sessions, with a maximum of 240
trials in each session. Proficient responding was characterized as correctly completing ~200 trials
per session. Each trial began with house light illumination after which rats had 3 s to enter the odor
port. Failure to enter the odor port resulted in restart of the trial. Once in the odor port rats were
required to hold for 1 s and upon exit had 3 s to enter the reward well. Again, failure to hold for 1 s or
enter the reward well within 3 s resulted in restart of the trial. On alternate days, rats were given
20-min ad libitum exposure to the untrained milk flavor.

Following implantation, rats were retrained on the initial odor and once single units were isolated the
unblocking procedure began. On the two learning days, rats received four trial types. The first was a
reminder of initial training. The remaining trial types began with a 200 ms presentation of the initial
odor and were followed by one of three 800 ms, novel yet distinguishable odors. The behavioral
requirements of each of these trial types were exactly as in initial training. Rats completed between
30-60 trials involving each novel odor per session. On the subsequent probe test day, rats received
a brief reminder of each trial type, ~10 total trials and then were presented the novel odors alone
without reward, interleaved with trials in which the initial odor was presented with reward, in order to
maintain responding. On the unrewarded, novel-odor extinction trials, the requirement to sample the
odor for 1-s and respond to the reward well was lifted. The unblocking procedure was repeated
seven to nine times per rat using a new set of blocked, number and flavor odors each time; in some
cases, a new initial odor was also trained prior to repeating unblocking. When the initial odor was
changed rats were trained on the new initial odor for 4-5 sessions prior to the first unblocking
procedure with that odor.

Consumption Tests: Consumption tests were given in a housing cage separate from their home
cage and experimental chamber. Two varieties of two-bottle tests were given. In the first,
consumption of one of the flavored milks (chocolate or vanilla) was compared to consumption of water. These tests were 10-min in duration and occurred on days when unblocking training was not performed. The second test directly compared consumption of the two flavored milks. These tests were 2-min in duration and occurred immediately following unblocking sessions. This second test was critical for showing that rats did not become selectively sated to the flavored milk they experienced more in the unblocking sessions. Further, these tests occurred immediately after unblocking sessions while rats were still in the recording setting, informing any preference that would have developed over the course of the unblocking session. For all tests the location of the bottles was swapped roughly every 20-30 seconds to equate time on each side.

Selective Conditioned Flavor Aversions: Naïve rats were exposed to the vanilla and chocolate-flavored milk twice each in one-hour sessions. During pre-exposure sessions intraperitoneal saline injections (0.9%, 5ml/kg i.p.) were given to habituate rats to the injection procedure. Conditioning consisted of five, one-hour exposures to each of the two solutions spaced over ten days. Exposure to the the devalued flavor was followed by injection of lithium chloride (0.3M LiCl, 5ml/kg i.p.). Exposure to the control flavor was followed by injection of saline. Consumption of each flavor was measured daily. A final 20-minute choice test was given in which both flavors were present. At the 10-minute mark the locations of the bottles were swapped. All factors (identity of devalued flavor, order of flavor presentation, side of flavor during choice test) were fully counterbalanced.

Single-Unit Recording: Neural activity was recorded using two identical Plexon Multichannel Acquisition Processor systems (Dallas, TX), interfaced with odor discrimination training chambers described above. After recovery from surgery, electrodes were advanced daily until activity was obtained on a majority of wires. During this process, rats received reminder training using the pre-trained initial odor, as described above. Once the electrode was in a suitable location in OFC, single units were isolated and rats showed proficient responding, the rat began unblocking. During
this three-day procedure, the electrode was generally left in the same position. Thus, although we
will not attempt to track neurons across sessions, the general population should be similar across
each three-day period. Following the completion of each three-day unblocking procedure, the
electrode was advanced 40-80 µm, and the process was repeated using new odor cues to test
neurons in a new location in OFC.

Statistical Data Analysis: Units were sorted using Offline Sorter software from Plexon Inc (Dallas,
TX) using a template matching algorithm. Sorted files were then processed in Neuroexplorer to
extract unit timestamps and relevant event markers. These data were subsequently analyzed in
Matlab (Natick, MA). To examine activity to the novel odors, we examined activity from 300-1300 ms
after initial odor onset, which corresponded approximately to the time during which the novel odors
were delivered to the odor port. To examine activity to the flavor outcome, we examined activity for
2000 ms starting with the first drop delivery. To examine activity to the number outcome, we
examined activity for 2000 ms starting 1000 ms following first drop delivery, coinciding with the third
drop delivery. The inter-trial interval was defined as the 2 s prior to illumination of the house light.

Normalized firing was calculated by taking the firing rate during a period of interest minus firing rate
during the ITI: Normalized firing = (Period spikes/s) – (ITI spikes/s). Neurons were identified as
being odor responsive with a Bonferroni-corrected t test (4 separate tests, 0.05/4 = 0.0125; p <
0.0125) comparing elevations in firing during each of the four odor epochs (initial, blocked, number
and flavor) from their respective ITIs. Neurons were classified as putative salience neurons if they
significantly increased firing to all odors; putative predictive neurons were classified by significantly
increasing firing to either or both the number and flavor odors, but not to all four odors. Single-unit
and population activity was plotted in 50-ms bins; population activity was analyzed with repeated
measures ANOVA with bin (50ms) and odor trial (initial, blocked, number and flavor) as factors.

Heat plots were constructed by calculating difference scores between normalized firing to Novel and
Initial odors (Figure 2F), Number and Blocked odors (Figure 3G) and Flavor and Blocked odors, in
200-ms sliding windows moving away from the novel odor onset in 50 ms increments. Warmer colors (dark red) indicated positive difference scores while cooler colors (dark blue) indicated negative difference scores. This was done for the first 10 trials of the identified population. Significance of differential firing to Novel and Initial odors (Figure 2F), Number and Blocked odors (Figure 3G) and Flavor and Blocked odors was determined by performing a one-tailed t test comparing differential firing to zero in the exact same 200-ms sliding windows for each of the 10 trials. Finally, for purposes of visualization only; single unit and population firing, as well as heat plot comparisons were smoothed by taking a four-bin average moving in 50 ms increments. This applied to Figure 2(A - bottom, B, and F), Figure 3 (A - bottom, B - bottom, C - bottom, D, G, I) and Figure 4A. All statistical analyses were performed on unsmoothed data.
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References


Figure Legends

Figure 1. Experimental outline, behavior summary and recording sites.

(A) Thirsty rats were initially trained to enter an odor port following illumination of a house light and respond at reward well below for two drops of flavored milk. Unblocking sessions consisted of 4 trial types. The first was a reminder of initial training. On the remaining three trial types, the originally trained odor was briefly presented followed by one of three novel odors. The reward following the novel odors was either unchanged (black; blocked trials), increased in number (blue; number trials), or its flavor was altered (green; flavor trials). Learning was assessed in a probe test in which the novel odors were presented in isolation, without reward. (B) Ten-minute consumption testing between chocolate & water, and vanilla & water on non-training days found that both were significantly and equally preferred to water (ANOVA, F_{1,5} > 5, p's <0.05). (C) Two-minute preference testing between chocolate and vanilla immediately following unblocking sessions found no flavor preference (t test, p>0.1). Scatter plot (right) shows preference for the trained flavor on each individual test (n=13). (D) Twenty-minute consumption testing from a separate group of rats (n=8) that received selective devaluation of one of the flavors found a significant difference in consumption between the non-devalued (Con) and devalued (Dev) flavors (t test, p<0.01). This was true for every rat tested (right). (E) Time in the reward well is plotted for the probe test trials. ANOVA for time spent in the reward well with odor (blocked, number and flavor) and trial (1-15) as factors found a significant odor x trial interaction (F_{1,47}=3.45, p<0.05). Planned comparisons confirmed that on the first 3 trials rats spent significantly more time in the reward well following number and flavor odors compared to blocked (p's<0.05) but responding to number and flavor did not differ (p>0.1). (F) Single unit activity was recorded from the lateral orbital and agranular insular cortices at roughly 3.2 mm anterior to bregma. *p<0.05; ns=not significant.
Preference for the devalued milk flavor (devalued)/(devalued + non-devalued) is shown for the two days of pre-exposure (P1-2), the five days of conditioning (C1-5, red background) and the final choice test (T). A value of 0.5 indicates equal preference while a value of 0.0 indicates an aversion to the devalued flavor. ANOVA for consumption over the five days of conditioning with session, flavor (choc vs. van) and treatment (control vs. devalue) found a significant session x treatment interaction (F4,28 = 10.91, p < 0.05) but no effect of or interaction with flavor. Asterisks indicate significance of a one-sample t test comparing % Devalue Preference to 0.5.

Figure 2. Single unit and population firing of putative salience neurons.

(A) Raster plots for firing of a single unit are shown for all initial (red), blocked (black), number (blue) and flavor trials (green). Odor on (On) is indicated by the first vertical line, onset of novel odor (Nov) by the second vertical line and odor offset (Off) by the third. Each tick represents a spike. Average activity across all trials for each odor is plotted (bottom). (B) Mean neural activity (novel odor epoch - ITI) for the putative salience neurons (n=55) is plotted. Line color as indicated in raster plots; shaded areas indicate standard error of mean. ANOVA with bin and odor as factors found significant effects of bin, odor and the bin x odor interaction (F1,54 > 2.0, p's < 0.01). ANOVA restricted to the novel odor period with odor and time (1st 500 ms vs 2nd 500 ms, shown in upper right inset) as factors found only a main effect of odor (F1,54 = 13.0, p < 0.01). Significant firing to the novel odors over the initial odor was observed throughout the novel odor period. (C) A scatter plot of novel odor firing versus initial odor firing is shown for putative salience neurons (n=55). A signed square root transformation of firing was used to best visualize population spread; all statistics were performed on non-transformed firing rates. Individual neurons showing significant differences in firing between the odors are outlined in black (t test, p < 0.05). A non-parametric sign test found significant, preferential firing to the
novel odors ($Z = 3.24, p < 0.01$). The population bias towards novel odor firing is apparent in the
bar histogram aligned to the diagonal axis; on which the difference score for each neuron is
plotted. Light grey bars represent units showing no differential firing; dark grey bars represent
units showing significant differential firing. (D) A scatter plot of predictive versus blocked odor
firing is shown. A sign test found no differential firing to the predictive and blocked odors by the
putative salience population ($Z = 0.54, p > 0.1$). (E) A scatter plot of flavor and number odor
differential firing is shown. A sign test found no differential firing to the number and flavor odors ($Z = 0.27, p > 0.1$). (F) Differential firing to the novel odors versus the initial odor on the first 10 trials of the
first unblocking day was calculated and plotted for the putative salience population (n=31).
Differential firing was calculated in a 300-ms sliding window for each 50-ms bin moving away
from novel odor onset: \(\text{mean} \left[ (\text{blocked odor} - \text{ITI}) + (\text{number odor} - \text{ITI}) + (\text{flavor odor} - \text{ITI}) \right] - \text{initial odor} - \text{ITI} \). The difference score for each bin was then plotted, with dark red bins
indicating maximal differential firing to the novel odors and dark blue indicating the opposite
pattern (y-axis shown on right of heat plot). (G) The significance of the increased firing to the
novel odors was determined by performing a one-tailed t test, comparing increases in
differential firing to 0, using a significance of $p < 0.05$ and a sliding window as in (F). Red bins
indicate significant elevations in firing to the novel odors over the initial odor.

Of the 37 units not analyzed in the main text, (A) 9 showed selective responding to the blocked
odor and (B) 9 showed selective responses to the initial odor. The remaining neurons showed
responses to different combinations of odors. (C) Neuron fired maximally to the number and
blocked odors, possibly signaling predicted reward flavor, independent of number. (D) Neuron
fired maximally to the flavor and blocked odors, possibly signaling predicted reward number,
independent of flavor. However, these patterns were rare and were not reflective of the odor-responsive population.

Supplemental 2 for Figure 2.

An identical analysis of only day 1 salience neurons (n=31) revealed nearly identical patterns as those reported when both day 1 and 2 neurons were analyzed. (A) ANOVA and subsequent post-hoc tests revealed identical significant results as in Fig. 2B of the main text. (B) Sign test for novel vs. initial odor firing differed slightly in it only approached significance (p = 0.07). (C,D) All other comparisons for salience neurons were identical to those reported in the main text.

Supplemental 3 for Figure 2.

Analyses identical to those performed in Fig. 2f,g of the main text, in which mean firing to all three novel odors was analyzed, were performed for each individual novel, odor. Separate analyses of temporal firing to (A,B) blocked vs. initial odor firing (C,D) number vs. initial odor firing and (E,F) flavor vs. initial odor firing revealed nearly identical patterns. Maximal responding is observed early on trial 1 and no or diminished responding is observed on subsequent trials.

Supplemental 4 for Figure 2.

Analyses identical to those in Fig. 2F,G of the main text were performed for salience neurons on unblocking day 2. (A) Salience neurons on unblocking day 2 did not show the same temporal response to the first presentation of novel odors. The white arrow indicates where maximum response was observed in unblocking day 1 neurons. (B) This description is confirmed by statistical analysis which found no significant increase in firing on the first presentation of day 2; but did find significance later in the odor period on subsequent trials.
Figure 3. Single unit and population firing of putative predictive neurons.

Single units plotted exactly as in Fig 2A showing (A) selective firing to the number odor (B) selective firing to the flavor odor (C) firing to both number and flavor odors. (D) Mean neural activity (novel odor epoch - ITI) for the putative predictive neurons (n=43) is plotted. Meaning of line colors and shading is maintained. ANOVA with bin and odor as factors found significant effects of bin, odor and the bin x odor interaction (F_{1,42} > 2.0, p's < 0.01). ANOVA restricted to the novel odor period with odor and time (1st 500 ms vs 2nd 500 ms, shown in upper right inset) as factors found a main effect of odor (F_{1,42} = 58.5, p < 0.01) and an odor x time interaction (F_{1,42}= 3.9, p < 0.05). At both times firing to the predictive odors was significantly greater than the blocked and initial odors; blocked firing was greater than initial firing only in the first half. (E) A scatter plot comparing firing to the predictive odors (signed square-root transform) versus the blocked odor is shown for the predictive population (n=43). Within this population there were three kinds of neurons based on firing versus ITI: number only (blue), flavor only (green) or flavor and number (purple). A sign test found significant, preferential firing to the predictive odors (Z = 4.88, p(s) < 0.01). Across all neurons there was zero correlation between predictive odor firing and blocked odor firing (R^2 = -0.01, p(r) = 0.39). (F) A scatter plot comparing firing to the flavor and number odors is shown for the predictive population (n=43). While some neurons did show differential firing (outlined in black) to either the number (n=14) or flavor (n=4) odor a sign test found no bias in firing to the number or flavor odor across the entire population. (Z=0.60, p>0.1). Across all neurons there was a highly significant, positive relationship between number and flavor odor firing (R^2=0.88, p<0.01). (G) Differential firing to the number odor versus the blocked odor on the first 10 trials of the first unblocking day was calculated and plotted for the number-responsive units within the predictive population (n=21). This was done in as in (Fig. 2F) except that the difference score was calculated as: (number odor - ITI) – (blocked odor
- ITI). (H) Significance for increased firing to the number odor over the blocked odor was calculated as in (g). Red bins indicate significant elevations in firing to the number odor over the blocked odor. Blue bins would indicate significant decreases in firing to the number odor below the blocked odor. (I) Differential firing to the flavor odor versus blocked odor on the first 10 trials of the first unblocking day was calculated and plotted for flavor-responsive units within the predictive population (n=18) as was done in (g). (J) Significance of differential firing calculated and displayed as in (H).

Supplemental 1 for Figure 3.

An identical analysis of only day 1 predictive neurons revealed identical patterns as those reported when both day 1 and 2 neurons were analyzed. (A) ANOVA, and subsequent post-hoc tests, revealed identical significant results as those reported in Fig 3D. (B) Sign test comparing predictive odor firing to blocked odor firing revealed a significant bias towards the predictive odors (p<0.05). (C) Sign test comparing flavor and number odor firing found no population bias towards either odor (p > 0.1).

Figure 4. Outcome selectivity of predictive neurons.

(A) For each predictive neuron that significantly increased firing to the flavor odor (total n=31; flavor-only n=6 [green]; number & flavor n=24 [purple]) we plotted its selective firing to the flavor odor (x-axis; [normalized flavor odor firing - mean(normalized initial odor firing, normalized blocked odor firing, normalized number odor firing)]) against its selective firing to the flavor outcome (y-axis; [normalized flavor outcome firing - mean(normalized initial outcome firing, normalized blocked outcome firing, normalized number outcome firing)]). Comparison of odor firing of single neurons to the population found a single outlier (neuron firing was 3 stdev > population firing). The outlier was omitted from this analysis. There was a significant, positive
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relationship such that greater selective firing to the flavor odor was associated with greater
selective firing the flavor outcome ($R^2=0.41$, $p<0.01$). (B) This relationship was restricted to the
flavor outcome; plotting selective flavor odor firing against selective number outcome firing
revealed zero correlation ($R^2=0.01$, $p=0.70$; calculation identical to A only mean(blocked, initial
and flavor outcome firing) was subtracted from number outcome firing. (C) For each predictive
neuron that significantly increased firing to the number odor (total $n=37$; number only $n=12$
[blue]; number & flavor $n=23$ [purple]) we plotted it’s selective firing to the number odor (x-axis;
[normalized number odor firing - mean(normalized initial odor firing, normalized blocked odor
firing, normalized flavor odor firing)]) against its selective firing to the number outcome (y-axis;
[normalized number outcome firing - mean(normalized initial outcome firing, normalized blocked
outcome firing, normalized flavor outcome firing)]). Two neurons showed selective odor firing 3
stdev above the population mean and were excluded from analysis. There was a significant,
positive relationship such that greater selective firing to the number odor was associated with
greater selective firing the number outcome ($R^2=0.27$, $p<0.01$). (D) This relationship was
restricted to the number outcome; plotting selective number odor firing against selective flavor
outcome firing revealed zero correlation ($R^2=0.04$, $p=0.26$) calculation identical to C only
mean(blocked, initial and number outcome firing) was subtracted from flavor outcome firing.
Finally, these statistical patterns were maintained if the flavor-only and number-only neurons
were analyzed in isolation: (A) $R^2 = 0.71$, $p = 0.03$, (B) $R^2 = 0.01$, $p = 0.82$, (C) $R^2 = 0.48$, $p =
0.01$ and (D) $R^2 = 0.18$, $p = 0.17$. Flav = flavor, Num = number, N&F = number and flavor.
Figure 1. Experimental outline, behavior summary and recording sites.

(A) Thirsty rats were initially trained to enter an odor port following illumination of a house light and respond at reward well below for two drops of flavored milk. Unblocking sessions consisted of 4 trial types. The first was a reminder of initial training. On the remaining three trial types, the originally trained odor was briefly presented followed by one of three novel odors. The reward following the novel odors was either unchanged (black; blocked trials), increased in number (blue; number trials), or its flavor was altered (green; flavor trials). Learning was assessed in a probe test in which the novel odors were presented in isolation, without reward.

(B) Ten-minute consumption testing between chocolate & water, and vanilla & water on non-training days found that both were significantly and equally preferred to water (ANOVA, F_1,5 > 5, p’s<0.05). (C) Two-minute preference testing between chocolate and vanilla immediately following unblocking sessions found no flavor preference (t test, p>0.1). Scatter plot (right) shows preference for the trained flavor on each individual test (n=13). (D) Twenty-minute consumption testing from a separate group of rats (n=8) that received selective devaluation of one of the flavors found a significant difference in consumption between the non-devalued (Con) and devalued (Dev) flavors (t test, p<0.01). This was true for every rat tested (right). (E) Time in the reward well is plotted for the probe test trials. ANOVA for time spent in the reward well with odor (blocked, number and flavor) and trial (1-15) as factors found a significant odor x trial interaction (F_1,47=3.45, p<0.05). Planned comparisons confirmed that on the first 3 trials rats spent significantly more time in the reward well following number and flavor odors compared to blocked (p’s<0.05) but responding to number and flavor did not differ (p>0.1). (F) Single unit activity was recorded from the lateral orbital and agranular insular cortices at roughly 3.2 mm anterior to bregma. *p<0.05; ns=not significant.
Figure 2. Single unit and population firing of putative salience neurons.

(A) Raster plots for firing of a single unit are shown for all initial (red), blocked (black), number (blue) and flavor trials (green). Odor on (On) is indicated by the first vertical line, onset of novel odor (Nov) by the second vertical line and odor offset (Off) by the third. Each tick represents a spike. Average activity across all trials for each odor is plotted (bottom). (B) Mean neural activity (novel odor epoch - ITI) for the putative salience neurons (n=55) is plotted. Line color as indicated in raster plots; shaded areas indicate standard error of mean. ANOVA with bin and odor as factors found significant effects of bin, odor and the bin x odor interaction (F1,54 > 2.0, p's < 0.01). ANOVA restricted to the novel odor period with odor and time (1st 500 ms vs 2nd 500 ms, shown in upper right inset) as factors found only a main effect of odor (F1,54 = 13.0, p < 0.01). Significant firing to the novel odors over the initial odor was observed throughout the novel odor period. (C) A scatter plot of novel odor firing versus initial odor firing is shown for putative salience neurons (n=55). A signed square root transformation of firing was used to best visualize population spread; all statistics were performed on non-transformed firing rates. Individual neurons showing significant differences in firing between the odors are outlined in black (t test, p < 0.05). A non-parametric sign test found significant, preferential firing to the novel odors (Z = 3.24, p < 0.01). The population bias towards novel odor firing is apparent in the bar histogram aligned to the diagonal axis; on which the difference score for each neuron is plotted. Light grey bars represent units showing no differential firing; dark grey bars represent units showing significant differential firing. (D) A scatter plot of predictive versus blocked odor firing is shown. A sign test found no differential firing to the predictive and blocked odors by the putative salience population (Z = 0.54, p > 0.1). (E) A scatter plot of flavor and number odor firing is shown. A sign test found no differential firing to the number and flavor odors (Z = 0.27, p > 0.1). (F) Differential firing to the novel odors versus the initial odor on the first 10 trials of the first unblocking day was calculated and plotted for the putative salience population (n=31). Differential firing was calculated in a 300-ms sliding window for each 50-ms bin moving away from novel odor onset: (mean [(blocked odor - ITI) + (number odor - ITI) + (flavor odor - ITI)] - initial odor - ITI). The difference score for each bin was then plotted, with dark red bins indicating maximal differential firing to the novel odors and dark blue indicating the opposite pattern (y-axis shown on right of heat plot). (G) The significance of the increased firing to the novel odors was determined by performing a one-tailed t test, comparing increases in differential firing to 0, using a significance of p < 0.05 and a sliding window as in (F). Red bins indicate significant elevations in firing to the novel odors over the initial odor.
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(B) This relationship was restricted to the flavor outcome; plotting selective flavor odor firing against selective number outcome firing revealed zero correlation ($R^2=0.01$, $p=0.70$; calculation identical to A only mean(blocked, initial and flavor outcome firing) was subtracted from number outcome firing. (C) For each predictive neuron that significantly increased firing to the number odor (total n=37; number only n=12 [blue]; number & flavor n=23 [purple]) we plotted it’s selective firing to the number odor (x-axis; [normalized number odor firing - mean(normalized initial odor firing, normalized blocked odor firing, normalized flavor odor firing)]) against its selective firing to the number outcome (y-axis; [normalized number outcome firing - mean(normalized initial outcome firing, normalized blocked number firing, normalized flavor odor firing]). Two neurons showed selective odor firing 3 stdev above the population mean and were excluded from analysis. There was a significant, positive relationship such that greater selective firing to the number odor was associated with greater selective firing the number outcome ($R^2=0.27$, $p<0.01$). (D) This relationship was restricted to the number outcome; plotting selective number odor firing against selective flavor outcome firing revealed zero correlation ($R^2=0.04$, $p=0.26$) calculation identical to C only mean(blocked, initial and number outcome firing) was subtracted from flavor outcome firing. Finally, these statistical patterns were maintained if the flavor-only and number-only neurons were analyzed in isolation: (A) $R^2 = 0.71$, $p = 0.03$, (B) $R^2 = 0.01$, $p = 0.82$, (C) $R^2 = 0.48$, $p = 0.01$ and (D) $R^2 = 0.18$, $p = 0.17$. Flav = flavor, Num = number, N&F = number and flavor.