

Orbitofrontal Ensemble Activity Monitors Licking and Distinguishes Among Natural Rewards

Ranier Gutierrez,¹ Jose M. Carmena,^{1,4} Miguel A. L. Nicolelis,^{1,2,3,4} and S. A. Simon^{1,2,4}

¹Departments of Neurobiology, ²Biomedical Engineering, and ³Psychological and Brain Sciences and ⁴Center for Neuroengineering, Duke University Medical Center, Durham, North Carolina

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Gutierrez, Ranier, Jose M. Carmena, Miguel A. L. Nicolelis, and S. A. Simon. Orbitofrontal ensemble activity monitors licking and distinguishes among natural rewards. *J Neurophysiol* 95: 119–133, 2006. First published August 24, 2005; doi:10.1152/jn.00467.2005. The classification of rhythmic licking into clusters has proved to be useful for characterizing brain mechanisms that modulate the ingestion of natural rewards (sucrose and water). One cortical area that is responsive to rewarding stimuli is the orbitofrontal cortex (OFC). However, it is not presently known how OFC neurons respond while rodents freely lick for natural rewards and whether these responses are related to the structure of licking clusters. We addressed these issues by showing that temporary inactivation of the OFC decreases the duration and increases the number of clusters and that the activity of OFC neurons changed at precise times before, during, and after the cluster terminates. Furthermore, analysis of the activity of OFC neuronal ensembles showed that they could discriminate cluster onset from termination, predict when a behaving animal will begin a cluster, and distinguish and anticipate between natural rewards. These results provide a new role for the OFC in influencing licking clusters and anticipating specific rewards.

INTRODUCTION

The orbitofrontal cortex (OFC) is involved in many functions that include the processing of almost all forms of rewarding stimuli (Hikosaka and Watanabe 2000; O'Doherty et al. 2001; Schoenbaum and Eichenbaum 1995a; Schoenbaum et al. 1998; Simmons et al. 2005; Tremblay and Schultz 1999; Wallis and Miller 2003; Zald et al. 2002). Orbitofrontal neurons are frequently multimodal in that they receive information from all sensory modalities and from brain areas involved in the evaluation of the hedonic properties of food and the motivation to eat or drink (Pickens et al. 2003; Yan and Scott 1996). In rats, the OFC has been shown to be involved in licking behavior because lateral OFC lesions alter mouth movements and tongue protrusions (Shiple et al. 1980; Wishaw and Kolb 1983). In addition, electrical stimulation of the lateral OFC induces tongue protrusions and rhythmical jaw movements like those observed during licking (Neafsey et al. 1986).

Licking plays important biological roles in grooming, rearing, sexual behavior, and the ingestion of liquids (Travers et al. 1997). During the ingestion of liquids, licking behavior occurs when a motivated animal actively identifies a liquid reward that would alleviate the physiological states of thirst or hunger. Licking consists of stereotyped rhythmic tongue movements that require the precise temporal coordination of muscles involved in swallowing, as well as jaw and tongue movements

(Welsh et al. 1995). The electrophysiological activity that underlies licking has been extensively studied at brain stem levels where complete sensory motor circuits are present in a central pattern generator located in the lateral reticular formation (Travers et al. 1997). In contrast, much less is known regarding how cortical neurons, in general, and ensembles of orbitofrontal neurons, in particular, respond when an animal licks freely for natural rewards.

When rodents lick for natural rewards, such as a solution of sucrose, their licks are not continuous but rather are separated by occasional short pauses. The licks that are demarcated by these pauses are called clusters (Davis and Smith 1992). From an empirical standpoint, the operational definition of licking clusters has been used to study brain mechanisms that regulate appetite, such as palatability and the physiological state of satiety. Specifically, increases in tastant palatability have been found to increase cluster duration, and on the approach to satiety, the number and duration of clusters decreases. Unlike licking itself, which is rhythmical and highly stable, licking clusters represent a goal-directed behavior in which the animal initiates and controls the length of the cluster.

In this study, we investigated the relationship between OFC activity and licking clusters by inactivating the OFC using muscimol, a GABA agonist, and by analyzing the activity of neuronal ensembles in the OFC obtained from freely licking rats. For the following reasons, we used chronic, multielectrode recording techniques to record from ensembles of OFC neurons. First, because licking involves the integration of chemosensory, motor and motivational responses, and neuronal ensemble recordings allow us to describe the full diversity of individual neuronal responses that occur simultaneously. Second, OFC neuronal ensembles offer a better alternative than single neuron recordings at predicting a behavioral event in a single trial basis (Cohen and Nicolelis 2004). A similar approach has been successfully used in the motor and somatosensory system (Carmena et al. 2003; Costa et al. 2004; Foffani and Moxon 2004; Ghazanfar et al. 2000; Laubach et al. 2000; Narayanan et al. 2005; Wessberg et al. 2000). However, to our knowledge, only a few studies have reported the ensemble activity of orbitofrontal neurons (Schoenbaum and Eichenbaum 1995b). We found that inactivation of the OFC modifies the structure of licking clusters and that the ensemble activity in OFC is composed of neurons that are recruited at precise times around the cluster while rats ingest palatable solutions. We have also found that neuronal ensembles perform better

Address for reprint requests and other correspondence: R. Gutierrez, Dept. of Neurobiology, Duke Univ. Medical Center, Bryan Research Bldg., Durham, NC 27710 (E-mail: gutierrez@neuro.duke.edu).

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than single neurons when predicting behavioral events associated with licking.

METHODS

Subjects and surgery

Five male Long-Evans rats (300–350 g) were anesthetized using 5% halothane followed by intraperitoneal injections of pentobarbital sodium (50 mg/kg) and 0.1 ml atropine sulfate. Movable electrode bundles comprised of 16 formvar-coated tungsten wires (15 μm diam) were bilaterally implanted above the lateral OFC at AP = 3.2, L = ± 3.2 , and DV = 4 mm from Bregma (Paxinos and Watson 1998; Schoenbaum et al. 1999), and the electrodes were advanced into the OFC. In addition, at the same coordinates, stainless steel guide cannulae (26 gauges) were bilaterally implanted above the lateral OFC of three different male rats. The cannulae were fixed with dental cement, and wire stylets were inserted in the guide cannula to prevent them from being blocked. The Duke University Institutional Animal Care and Use Committee approved all protocols.

Licking behavior detection

All the experiments were performed in an operant box enclosed in a ventilated and sound-attenuating cubicle. Each box contained a lickometer (V shape, vertical slot) with a photo-beam sensor (MedAssociates) that was used to register the times when the rat's tongue contacted the drinking tubing (10-ms resolution). Under these conditions, the amount of tastant received in each lick was $\sim 5\text{--}6\ \mu\text{l}$. The times the animal licked and the neural activity were recorded simultaneously. The analysis of licking was performed following the work of Davis and Smith (1992). That is, a train of at least three rhythmic licks was assigned to the same cluster when their interlick interval was $< 500\ \text{ms}$. For the electrophysiological recordings, only a small portion of the clusters were smaller than three or four licks (per *experiment 4* of about 80 clusters). Because of this low frequency, the inclusion or exclusion of such small clusters did not significantly modify any of the results.

Behavioral procedure (neurophysiology)

One week after surgery, the animals were habituated for 1 h in an operant chamber for 2 days. They were then placed on a 23.5-h water deprivation schedule, whereupon for 2 additional days they were free to drink water from a sipper tube for 30 min. A total of 19 experiments were conducted. In 16, the water-deprived rats were allowed 15 min to drink 10 ml of water, and after a 5 min pause, they were given access to 0.4 M sucrose until they stopped drinking for $> 10\ \text{min}$. In the other three experiments, they were given only 0.4 M sucrose. The decision to give water before sucrose was made to avoid a negative reward contrast effect, where rats did not ingest water when they had already ingested sucrose.

Recording neural activity

Neural activity was recorded from the implanted microwires and processed using a Multineuron Acquisition Processor (Plexon, Dallas, TX). Only single neurons with action potentials of $> 3:1$ signal-to-noise ratios were analyzed. The action potentials were isolated on-line by means of voltage-time threshold windows and a three principal components contour templates algorithm. A cluster of waveforms was assigned as a single unit only when both interspike intervals (ISIs) were larger than the refractory period, set to 1.5 ms, and when the three-dimensional (3-D) projection of the first three principal components formed a visible cloud (Kralik et al. 2001). Only time-stamps from off-line sorted waveforms were analyzed. From five rats, a total

of 172 single units were obtained in 19 experiments. The average number of neurons recorded from the same electrode was 1.36.

Behavioral procedure (microstructure of licking) and microinjection of muscimol

One week after surgery, the three animals with cannulae implanted were handled daily and habituated for 1 h in an operant chamber for 2 days. They were then placed on a 22-h water deprivation schedule, whereupon for 2 additional days they were free to drink 0.4 M sucrose from a sipper tube for 2 h. The animals were permitted to drink at the same time each day. On the third day, 10 min before they usually drank sucrose, the animals received bilateral OFC injections of muscimol (0.5 μg) dissolved in saline. This dose of muscimol, injected in the OFC, was previously shown to abolish reversal learning of an odor discrimination task (Kim and Ragozzino 2005). As controls, 2 or 3 days after muscimol infusion, the same rats received injections of sterile 0.9% saline into the lateral OFC 10 min before sucrose intake. In these three drinking sessions, licking clusters were analyzed, and the probability density function of the cluster's duration was calculated using a Gaussian kernel (computed with the Matlab function *ksdensity*; Fig. 1C).

Microinjections of saline or muscimol were given bilaterally through a 30-gauge stainless steel injector 1.8 mm longer than the tip of the guide cannula. The injector was connected by Teflon tubing to a 10- μl glass syringe attached to a microinfusion pump (CMA/Microdialysis AB). Infusions of 0.5 μl were given per hemisphere over 2 min. The injector was left inside the guide cannula for 1 additional min to allow diffusion.

Histology

At the end of the experiments, each rat was injected with an overdose of pentobarbital sodium (150 mg/kg) and perfused with saline, followed by 0.4% formalin. The brains were removed and placed in 30% sucrose/PBS (vol/vol) solution. Brains were sectioned (50 μm) and stained with cresyl violet to establish the track of electrodes.

Data analysis

CUMULATIVE SUM WITH BOOTSTRAPPING. This procedure has been described elsewhere (Wiest et al. 2005). Briefly, statistically significant responses were identified from the poststimulus time histograms (PSTHs) using cumulative deviations from baseline firing rates. To identify the onsets of significant deviations, we calculated the deviation D_n of the poststimulus cumulative summed firing rate from the expected cumulative sum, based on the average baseline firing rate λ , at each poststimulus bin i

$$D_n = \sum_{i=1}^n \text{Post}_i - \sum_{i=1}^n \lambda_i$$

Here Post_i is the observed firing rate in poststimulus bin i .

To assess the significance of deviations from the expected cumulative sum, we made 1,000 bootstrapped samples (with replacement) using the baseline firing rates. An empirical distribution was constructed by calculating the deviations of the samples' cumulative summed firing rate from the expected cumulative sum based on λ . This empirical distribution was used to find the poststimulus bin (if any) at which the cumulative sum of the poststimulus firing rate exceeded 99% of the cumulative firing rate from the empirical baseline distributions (Martinez and Martinez 2002). This bin was recorded as the onset of an excitatory or inhibitory response. The response offset was identified as the first zero crossing of the derivative of the deviation from the baseline firing rate (D_n). This procedure identified the times at which the PSTH returned to its baseline firing rate.

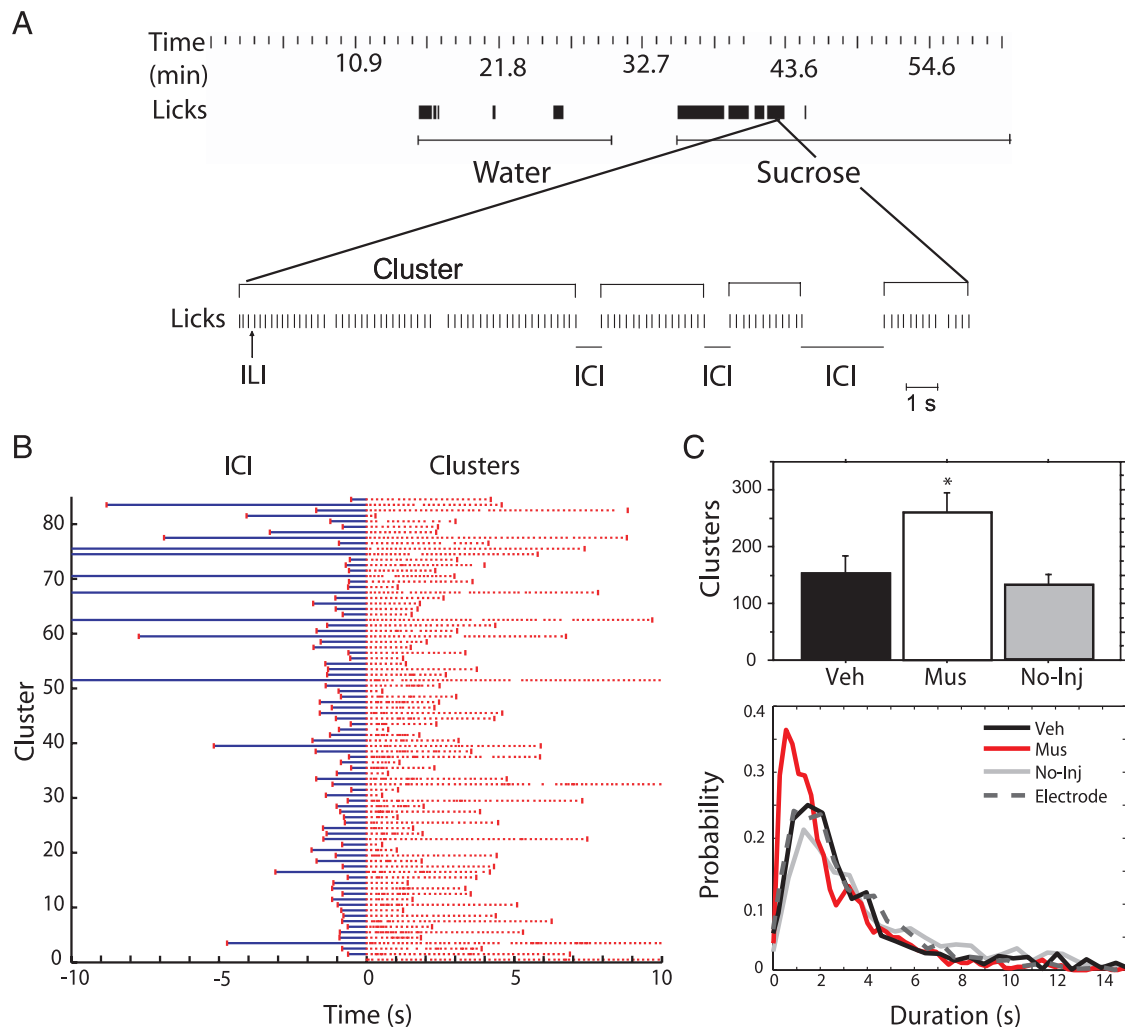


FIG. 1. Analysis of the microstructure of rats licking for sucrose. *A*: all licks (black marks) recorded in an experiment where a rat first freely licked for water and later for 0.4 M sucrose. *Inset*: 23-s segment of this experiment showing that licking is a highly stereotypic behavior with a nearly constant interlick interval (ILI). A cluster is defined as a collection of licks separated by pauses >500 ms; these pauses are named intercluster intervals (ICIs). *B*: perievent raster plot of clusters and ICIs (blue horizontal lines) in the original order (from *bottom* to *top*) of a single experiment where a rat drank sucrose. The 1st lick in a cluster was aligned to time = 0 s. Each red mark represents the time when a lick occurred (larger red mark depicts the last lick in a cluster). To better show the ICIs that preceded each cluster, the last lick in a cluster was also plotted before 0 s (see red mark in ICI section). For visualization purposes, the abscissa was truncated at ± 10 s. *C*: effects of muscimol inactivation of the orbitofrontal cortex (OFC) on the microstructure of licking clusters. *Top*: average number of clusters from rats after injection into the OFC of vehicle (Veh), muscimol (Mus), or no injection (No-inj). Rats had access to sucrose for 2 h. *Significantly different relative to vehicle and no injection treatment ($P < 0.05$). *Bottom*: probability density function of clusters' duration. It is seen that inactivation of the OFC increases the probability of clusters with short (≤ 1 s) durations. This plot also shows, for comparison, distribution of 1,939 clusters observed while neuronal activity was simultaneously recorded in 5 other rats with electrodes in the OFC (dashed line).

In those cases where a PSTH contained more than one significant change in firing rate, only the first significant response was counted. We describe below how we selected the baselines for cluster initiation and termination. *Cluster initiation* was when rasters and their corresponding PSTH of the activity ± 500 ms around the first lick in a cluster were analyzed. Because a large majority of OFC neurons showed anticipatory activity, the baseline was set from -500 to -250 ms before cluster start. *Cluster termination* was when rasters and their associated PSTHs of the ± 500 ms around the last lick in a cluster were analyzed. The activity from -500 to 0 ms before cluster termination was used as a baseline. Note that in this condition, an excitatory or inhibitory response means a significant increase or decrease in activity when rats stop licking, respectively.

LICKING-RELATED ACTIVITY. This type of response was quantified by performing a cross-correlation analysis between neuronal activity and licking. All the licks recorded in an experimental session were

used for the analysis seen in Fig. 4 and Table 1 (licking activity). To assess whether the cross correlations were statistically significant, 250 surrogates of clusters were simulated by using scrambled positions of the real clusters [intercluster interval (ICI) > 500 ms] relative to neural activity. Subsequently, by using the surrogate positions of clusters, 250 PSTHs were constructed (-200 to 200 ms around each single lick, bin = 10 ms). Because the real interlicks intervals in the clusters were used, these surrogates maintained the rhythmical structure of licking. A significant P value ($\alpha = 0.01$) for every bin was calculated by counting the times that the activity in the real PSTH exceeded 99% of surrogates PSTHs. This method determines if oscillatory activity related to licking was significantly above the background firing rate. In addition, the rate histograms were used to estimate the power spectra of the spike trains during licking. Only neuronal activity that showed both a peak at the same frequency of licking (6–7 Hz) and a significant cross-correlation with licking was named licking-related activity.

TABLE 1. *OFC activity induced by licking for sucrose*

	Exc	Inh	Total	Single Response	Complex Responses		
Ant	59	12	71	Ant (23 + 4)	Ant and Term (22 + 7)	Ant and Lick (7 + 0)	Ant, Lick and Term (1 + 7)
DS	13	25	38	DS (4 + 5)	DS and Term (2 + 17)	DS and Lick (1 + 3)	DS, Lick and Term (6 + 0)
Lick	—	—	30	Lick (4)	Lick and Term (0 + 1)		
Term	41	31	72	Term (5 + 4)			

In parenthesis, number of neurons with excitatory and inhibitory activity, respectively. Except for “Term” and “Lick and Term,” inhibitory and excitatory activity was calculated during the cluster start. Exc, excitatory activity; Inh, inhibitory activity; Ant, anticipatory activity; DS, during stimulus activity; Lick, licking related activity; Term, termination.

Ensemble activity analysis

CLASSIFICATION OF CLUSTER START AND TERMINATION. The PSTH-based classification method, developed by Foffani and Moxon (2004), was used to classify the beginning and the termination of a licking cluster. This classification method consists of creating two sets of templates (e.g., 1 for cluster initiation and 1 for cluster termination). The templates are the concatenation of the individual PSTHs of all the simultaneously recorded neurons in a row vector. Each template contains the average activation pattern of the ensemble evoked by a stimulus (see Fig. 7A). Once the templates were constructed, a single trial or testing trial was removed from only one template at a time and the Euclidean distance of the testing trial with respect to both templates was calculated. Finally, the testing trial was classified as the template with the smaller Euclidean distance. This procedure was continued until all the trials were classified (for cross-validation). For visualization purposes, the row vectors representing the template and testing trial were plotted in its matrix form of size [Bins by Neurons] (see Fig. 7A). Note that here the word “trial” means the activity (spikes/bin) of all the neurons recorded simultaneously when a single cluster start or end occurred.

The PSTHs were constructed by using the activity ± 500 ms around the first lick for cluster initiation and the activity ± 500 ms around the last lick of a cluster for cluster termination (bin size, 10 ms). Classification performance was measured as the percentage of testing trials that were correctly classified by the neuronal ensemble. The means \pm SE overall experiments per template size were reported. Statistical significance was computed with one group *t*-test (right-tail), where the alternative hypothesis was mean ensemble classification significantly greater than chance classification (50%).

CLASSIFICATION OF SUCROSE AND WATER. The PSTH-based classification method described above was also used to distinguish between licking for water or sucrose. In this case, one template was for sucrose and another for water. In 16 of 19 experiments, rats were presented with both water and sucrose. Because this method requires the same number of trials per stimulus, the same number of clusters with sucrose and water were used in the analysis. We only used clusters >500 ms, to ensure that rats were licking in all the clusters used in the analysis. Finally, the PSTH-based classification method was used in four 250-ms epochs (bin = 10 ms), around the cluster start of water and sucrose. For each epoch, the percentage classification performance was calculated per experiment, and the means \pm SE overall experiments per epoch was reported. As above, statistical significance was computed with one group *t*-test (right-tail).

NEURON-DROPPING ANALYSIS. To determine whether information was distributed within the ensembles of neurons recorded in each experiment, we used the method of randomly dropping neurons (Wessberg et al. 2000). Briefly, single neurons were randomly removed, one at a time, from the neuronal ensemble recorded in each session. The performance of the PSTH-based classification method was recalculated using the remaining neurons in the ensemble until only a single neuron remained. For each neuronal ensemble, this procedure was repeated 50 times to obtain an average curve that describes the classification performance as a function of ensemble

size. In addition, ranked neuron-dropping analysis was also performed to determine how neuronal ensemble performance was affected when the best single neurons, one at a time, were removed from the ensemble. In this analysis, the classification performance achieved by each individual neuron was ranked starting from the best performing single neuron and gradually removing one neuron at a time until only the worst predicting neuron in each ensemble remained in the sample. Only neuronal ensembles with >10 neurons and whose performance was above chance level (i.e., the neuronal ensembles that discriminated between sucrose and water) were used for these analyses ($n = 6$ ensembles, with 87 neurons in total). A similar analysis was performed for classification of the start and termination of the clusters (Fig. 7, *D* and *E*; $n = 9$ ensembles, with 118 neurons in total) and for linear prediction of cluster initiation (Fig. 8, *E* and *F*; $n = 7$ ensembles, with 92 neurons in total).

LINEAR PREDICTIONS OF CLUSTER INITIATION. The licking behavior of the animals was modeled as a weighted linear combination of neuronal activity using a multiple linear regression—a general description of which can be found in Haykin (2002). This linear model has the basic form of

$$\mathbf{y}(t) = \mathbf{b} + \sum_{u=-m}^n \mathbf{a}(u)\mathbf{x}(t-u) + \epsilon(t).$$

In this equation, $\mathbf{x}(t-u)$ is an input vector containing spikes per bin (50 ms) at time t for each time-lag u , $\mathbf{a}(u)$ is a vector of weights at time-lag u , \mathbf{b} is a vector of y -intercepts and $\epsilon(t)$ is the residual errors. The time-lags u in the summation can in general be negative (in the past) or positive (in the future) with respect to the present time t . Finally, $\mathbf{y}(t)$ is a binary vector of licking clusters (i.e., start of cluster) at time t . In this vector “1” was used for each bin where a cluster start occurred, “0” otherwise. This equation can be recast in matrix form as

$$\mathbf{Y} = \mathbf{X}\mathbf{A}$$

where each row in each matrix is a unit of time (or bin) and each column is a data vector. Note that matrix \mathbf{X} contains lagged data and thus has a column for each lag times each neural channel, e.g., 20 channels and 10 lags imply 200 columns. The y -intercept is handled by prepending a column of ones to matrix \mathbf{X} . Matrix \mathbf{A} is solved by

$$\mathbf{A} = \text{inv}(\mathbf{X}^T\mathbf{X})\mathbf{X}^T\mathbf{Y}.$$

Several time-lags preceding or succeeding a given point in time were used for training and testing the linear model. The optimal number of time-lags was found empirically deleted (Carmena et al. 2003; Wessberg and Nicolelis 2004), resulting in five negative and five positive time-lags. This is equivalent to 250 ms before and 250 ms after each present time t (see Fig. 8, *C*, *E*, and *F*). For the perievent analysis of ensemble performance (see Fig. 8*D*), only one lag at a time was used by the linear model to predict the onset of a licking cluster. Models were trained with 200 s (4,000 samples or bins) of continuous data and tested by applying them to subsequent testing windows of 100 s (2,000 samples). These parameters were found empirically as in Carmena et al. (2003).

To quantify the performance of the linear model, a threshold level was set on the \hat{y} estimates (neural predictions shown in Fig. 8, *A* and *B*, in red). \hat{y} values crossing the threshold at the time of the cluster start were considered as true positives, whereas \hat{y} values crossing the threshold at any other time were considered false positives (Fig. 8*A*, arrows). Two curves representing the percentage of true and false positives predictions were obtained for all possible thresholds levels. The maximum Euclidean distance between these curves determined the threshold used in all the testing windows of an experiment.

For each experiment, the means \pm SE percentage of true and false positive of all the testing windows was used as an individual ensemble performance index, and the overall means \pm SE across experiments was used as the global neuronal ensembles performance in the OFC (Fig. 8*C*). In 9 of the 19 experiments, it was not possible to perform this analysis because of an insufficient number of clusters to train and test the linear model. Therefore linear predictions were performed in the remaining 10 experiments. For a given experiment, the statistical difference of the true and false positive obtained in all the testing windows was calculated using Mann-Whitney test with an α of 0.05%.

To maximize signal-to-noise ratio, nonlicking epochs bigger than 2.5 s were removed before model fitting. This criterion assures that only the neuronal activity recorded during licking behavior was used to train and test the linear model excluding other kind of behaviors such as grooming, resting and sleeping.

RESULTS

Licking behavior

The licking behavior obtained from five implanted rats that licked for 0.4 M sucrose yielded an average licking frequency of 6.5 ± 0.002 Hz (range, 5.9–7.4 Hz; $n = 41,394$ licks). This rhythmic licking pattern was broken by a series of short pauses. When the pauses (i.e., the interlick interval) were >500 ms, the groups of licks were called clusters (see Fig. 1*A* and Davis and Smith 1992); using this criterion, a total of 1,939 clusters were analyzed: the median cluster duration, size, and ICI was 2.4 s, 17 licks, and 1.2 s, respectively. These results are in good agreement with previous studies (Davis and Smith 1992; Spector et al. 1998). Figure 1*B* shows the clusters and ICIs in an experiment where a rat licked for 0.4 M sucrose.

Inactivation of the OFC disrupts cluster structure

As discussed, the OFC is involved in licking and tongue protrusions. However, it is not known whether the activity from OFC may influence licking clusters. Therefore we functionally inactivated the lateral OFC (see Supplementary Fig. S1 for histology) 10 min before permitting the rats to freely lick for 0.4 M sucrose. The microstructure of licking was compared during three conditions: controls 1 day before injection (No-inj), the next day under muscimol inactivation (Mus), and a later day when a saline vehicle solution (Veh) was injected. Inactivation of the OFC with muscimol did not change the licking rate (No-inj = 7.0 ± 0.29 Hz, Mus = 7.1 ± 0.37 Hz, Veh = 6.6 ± 0.37 Hz; $F_{(2,6)} = 0.7$, $P > 0.05$) or the total number of licks (No-inj = 4279 ± 61 , Mus = 4647 ± 464 , Veh = 4353 ± 7 ; $F_{(2,6)} = 0.51$, $P > 0.05$). However, as seen in Fig. 1*C*, inactivation of the OFC increased the number of licking clusters (No-inj = 131 ± 17 , Mus = 261 ± 35 , Veh = 151 ± 31 ; $F_{(2,6)} = 5.7$, $P < 0.05$). A Fisher post hoc analysis indicated that only the injection of muscimol was significantly different from both No-inj and Veh ($P < 0.05$). In addition,

OFC inactivation also reduced the cluster duration (length of clusters) relative to vehicle and no-injection days (see Fig. 1*C*; $F_{(2,1628)} = 32.5$, $P < 0.0001$). These results indicate that the OFC activity may influence the microstructure of licking for sucrose, because its inactivation increased the number and decreased the duration of the clusters.

We also found that inactivation of the OFC reduced the total intake of sucrose (No-inj = 24 ± 0.3 ml, Mus = 17 ± 1.3 ml, Veh = 27 ± 1.4 ml; $F_{(2,6)} = 5.7$, $P < 0.05$). A Fisher post hoc analysis indicated that only muscimol injection was significantly different from both No-inj and Veh ($P < 0.05$). This reduction of sucrose intake, together with the constancy in the total number of licks, indicates that on OFC inactivation there was a reduction in the amount of liquid consumed for each lick. That is, the total intake/total licks was significantly reduced by muscimol treatment (No-inj = 6 ± 0.1 μ L/lick, Mus = 4 ± 0.1 μ L/lick, Veh = 6 ± 0.3 μ L/lick; $F_{(2,6)} = 37$, $P < 0.01$). Taken together, these results suggest that the OFC activity is important for an efficient use of the tongue during ingestion of sucrose.

OFC neuronal responses while freely licking for sucrose

The above behavioral results suggest that the OFC activity is important for the organization of licking clusters for natural rewards. For this reason, we decided to record the neuronal activity of small ensembles of OFC neurons while rats freely licked for sucrose or water.

A total of 172 OFC neurons obtained from five water-deprived rats were characterized during the ingestion of 0.4 M sucrose. We used the neuronal activity around the first or last lick in a cluster to identify a neural correlate of the cluster start or termination, respectively. Statistically significant changes in activity in the PSTH were determined using a cumulative sum statistic combined with a bootstrapping algorithm (Wiest et al. 2005). This analysis identified 123 neurons that had a total of 211 significant ($P < 0.01$) responses (changes in firing rate either excitatory or inhibitory relative to baseline) during one or more epochs related to the clusters. We named these epochs: anticipatory, during stimulus (DS), and termination. There is, in addition, licking-related activity that is described below in greater detail. Representative examples of these four major types of OFC neuronal responses are depicted in Fig. 2: anticipatory (Fig. 2, *A–C*), DS responses (Fig. 2, *D* and *F*), licking (Fig. 2*E*), and termination responses (Fig. 2, *G–I*).

Anticipatory responses (41%, 71/172) were those in which neuronal activity significantly changed ≤ 250 ms before the cluster start (Figs. 3, *A* and *B*). Although many anticipatory neurons had their maximum peak of activity before the start of the cluster ($n = 36$), other anticipatory neurons ($n = 22$) reached their maximum activity during the 200-ms interval after the first lick in a cluster (Fig. 3*C*). Most of anticipatory responses returned to the baseline firing rate in <400 ms after cluster start (Fig. 3*D*). Neurons that changed activity within the first 500 ms after cluster start were named “DS responses” (22%, 38/172; Fig. 3, *middle column*). Such neurons may be driven by taste or reward information. A subset of them may also be driven by somatosensory input, because their activity correlates with licking. Most DS responses had onsets of significant activity during the first 200 ms after cluster start, and their periods of excitation or inhibition usually continued

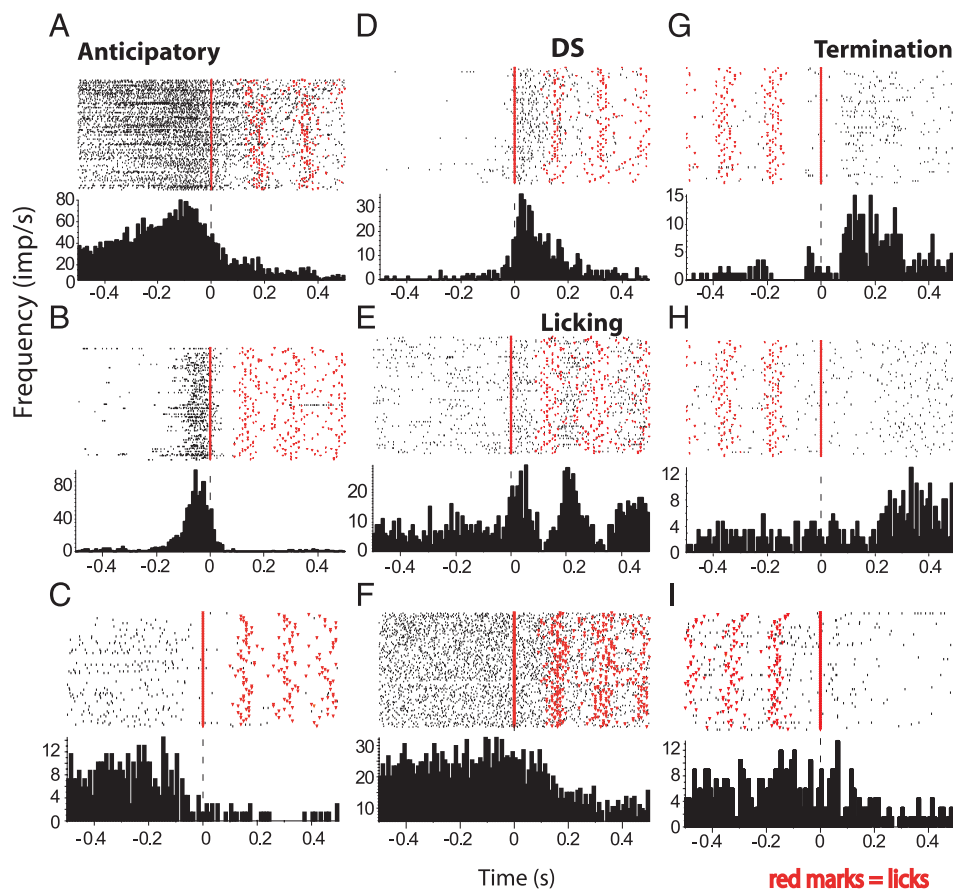


FIG. 2. Single neurons recorded in the OFC while rats lick for 0.4 M sucrose. *A–F*: rasters and peristimulus time histograms (PSTHs) of neuronal activity are aligned to the 1st lick in the clusters. In all panels, red dots in rasters denote times the animal licked. Neuronal activity shown in *A*, *B*, and *C* was denoted as anticipatory because they were active before onset of a cluster (at 0 s). *C*: representative example of inhibitory activity. Neuronal activity in *D* and *E* was labeled during stimulus (DS) and licking, respectively, because they were active after the 1st lick in the cluster. Note that the neuronal activity shown in *E* covaries with licking, whereas the neuron in *D* only responded after the 1st lick in the cluster. *F*: neuron with inhibitory activity during the DS epoch. Neuronal activity in *G–I* was called termination, because they changed their activity after the last lick in the cluster, which in these 3 panels, is also at time = 0 s.

>500 ms after cluster start. Termination responses (42%, 72/172) were those that exhibited significant changes in activity ≤ 500 ms after the last lick in the cluster (Fig. 3, right column). These responses frequently exhibited a peak in activity between 200 and 400 ms after the cluster ended. However, 18% ($n = 13$) of them exhibited a transient response during the termination epoch (Figs. 3*A* and 2*G*), suggesting that only a few neurons appear to show specific activity for signaling the end of a cluster.

Other less frequent classes of OFC neuronal patterns were also identified in our recordings. Licking responsive neurons (17%, 30/172) had activities that covaried with individual licking in the sense that neuronal firing tracked the licking frequency (6–7 Hz). These neurons exhibited a variety of responses. Specifically, a subpopulation of them (12/29, tested with both stimuli) oscillated at the same phase and magnitude for both sucrose and water (Fig. 4*A*). For this reason, it is likely that these neurons conveyed somatosensory information produced by tongue movements. In contrast, other oscillatory neurons (5/29) were only activated for one of the gustatory stimuli (sucrose or water; Fig. 4*B*). Yet another subset of OFC cells (12/29) could distinguish between these tastants by having a different peak phase (Fig. 4*C*) and/or different firing rates (Fig. 4, *C* and *D*). These latter neuronal responses are likely to be multimodal, and contain information about both mouth/tongue movements as well as the identity of the reward.

With the exception of licking-related activity, significant firing rate modulation was either excitatory or inhibitory (see Table 1). We observed that most OFC neurons were significantly activated (or inhibited) in multiple epochs. These were

named “complex” neurons (Fig. 5; Table 1). Because the cluster start and end were independently compared, it is possible that an anticipatory or DS neuron with a sustained increase in firing rate through the whole licking cluster was classified as a complex neuron (see Supplementary Fig. S3¹ on-line for the characterization of these responses).

OFC neuronal ensembles

Figure 5 shows the PSTHs of eight simultaneously recorded OFC neurons that were obtained during a single session while the animal drank (licked) either 0.4 M sucrose (Fig. 5*A*) or water (Fig. 5*C*). The ensemble is presented in this manner to show the activity around the cluster start and termination. This figure shows that the ensemble’s activity concomitant to the clusters contains neurons that may exhibit several firing rate modulations before, during, or after cluster termination. For example, neuron 6 responded to both cluster onset (Fig. 5*A*, left) and termination (Fig. 5*A*, right). This ensemble also contains neurons that only responded at a specific time, such as neuron 3 that only responded shortly after the first lick in the cluster (see Fig. 5, *A* and *B*). The various types of OFC activity observed while a rat licked for sucrose are seen in Table 1. These data show that some neurons respond only at a particular time with respect to the cluster indicating that their activity is unrelated to tongue movements or the tastant per se (because the tastant is received in every lick). Rather, the significant

¹ The Supplementary Material for this article (3 figures) is available online at <http://jn.physiology.org/cgi/content/full/000467.2005/DC1>.

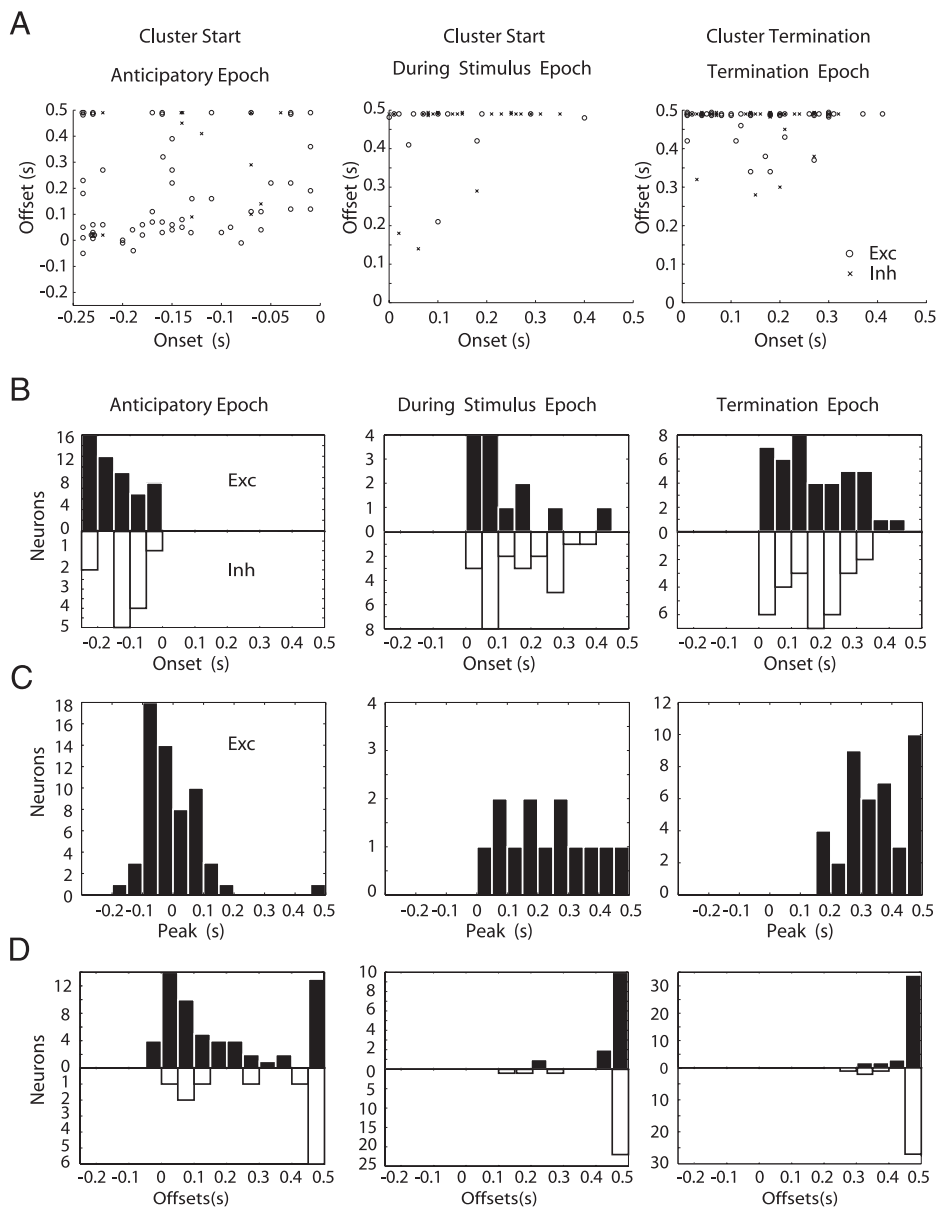


FIG. 3. Onsets, offsets, and peaks of significant OFC activity. *A*: scatter plot of onset vs. offset of statistically significant firing rate modulations. Each point represents a single neuron showing either excitatory (Exc "o") or inhibitory (Inh "x") activity during cluster start. Note that in the *left and middle columns*, time 0 corresponds to the 1st lick in a cluster. *Right panels* display onsets and offsets of neurons with significant firing rate modulations during the termination epoch. Here time 0 represents the last lick of the cluster. *B–D*: distributions of onsets, offsets, and maximum peaks histograms for anticipatory, DS, and termination responses (*left, middle, and right columns*, respectively). In each panel, solid bars indicate the number of excitatory neurons, whereas upside down and white bars depict inhibitory activity. Maximum peak of activity only applies to excitatory activity.

firing rate modulation of these neurons was related to a specific time within the cluster. In contrast, other neurons signal multiple epochs of the cluster.

Although some similarities can be observed in the activation pattern of the OFC neuronal ensembles when rats drank sucrose (Fig. 5A) or water (Fig. 5C), many differences were also evident, indicating, as shown below, that OFC neuronal ensembles may be used to discriminate between water and sucrose.

OFC neuronal ensemble activity and the classification of natural rewards: sucrose versus water

In both rodents and nonhuman primates, individual OFC neurons have been shown to distinguish among rewards (Hikosaka and Watanabe 2000; Schoenbaum et al. 1998; Thorpe et al. 1983; Tremblay and Schultz 1999; Watanabe 1996). Here we show that the information present in OFC neuronal ensembles can be used to discriminate between sucrose and water both as rats anticipate licking these tastants and for ~250 ms after they taste it. These

results were obtained using a PSTH-based classification method (Foffani and Moxon 2004). For a given tastant, each trial (cluster) was tested and assigned to the closest template (Fig. 6A). We first determined whether the ensemble activity could discriminate between sucrose and water using the activity ± 500 ms relative to the start of the cluster. The percentage of single trials correctly classified from all experiments was $62.3 \pm 3\%$ ($n = 16$), a value significantly above chance ($t_{(15)} = 3.6$, $P < 0.01$). One large ensemble ($n = 14$ neurons) achieved the best performance, which correctly classified 88% of the trials. Figure 6B shows that the probability of distinguishing between sucrose and water increases with the size of the ensemble ($r^2 = 0.45$, $F_{(1,14)} = 11.4$, $P < 0.01$).

The above discrimination did not arise from small difference in licking rates because the licking rate was 6.4 ± 0.002 Hz for 0.4 M sucrose and 6.5 ± 0.003 Hz for water (no significant, t -test, $t_{(df = 30)} = -0.52$, $P > 0.05$). More importantly, the absolute difference in licking rates (for individual sessions) did

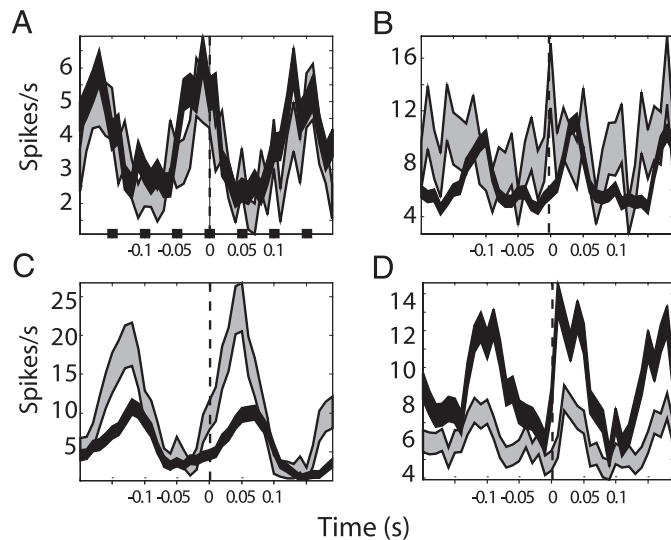


FIG. 4. Oscillatory activity in OFC neurons obtained while animals freely lick for water and sucrose. PSTHs of neural activity were aligned to when the animal contacted the sipper tube. **A:** neuronal activity of OFC neuron in which the amplitude and phase of the responses to sucrose (black) and water (gray) are the same. Note that the peak of activity occurred during tongue protrusion phase, i.e., 30 ms before the tongue made contact with sipper tube (*time 0*). **B:** neuron exhibiting oscillatory activity during sucrose intake but not during licking water. **C:** neuron that exhibits different firing rates (magnitude) and peak phase to water and sucrose. **D:** neuron that exhibits different firing rates for water and sucrose but has the same peak phase. Width of PSTHs represents SD calculated with bootstrapping.

not correlate with the classification performance ($r^2 = 0.09$; nonsignificant $F_{(1,15)} = 1.53$, $P = 0.23$). Therefore the ensemble discrimination of water and sucrose was induced by something else than any difference in licking rate.

To determine the time (relative to the start of a cluster) at which there was sufficient information to distinguish between sucrose and water (Fig. 6C), the neuronal activity was partitioned into four epochs: baseline (-500 to -260 ms), approach (-250 to -10 ms), and two periods of drinking (D) (0 – 250 and 260 – 500 ms). In the baseline (B) epoch, the performance across experiments ($53 \pm 2\%$, $n = 16$) was not significantly different from chance ($t_{(15)} = 1.47$, $P > 0.05$). However, OFC neuronal ensembles could be used to discriminate between sucrose and water during the anticipatory (A) epoch, that is, 250 ms before the rat's tongue made contact with the tastants ($60 \pm 3\%$, $t_{(15)} = 3.02$, $P < 0.01$). Importantly, the ensemble's neuronal activity could distinguish between the two tastants after the first and second lick (Fig. 6C). That is, for the two drinking (D) epochs, the percentage of correct classifications were $59 \pm 3.5\%$ ($t_{(15)} = 2.4$, $P < 0.05$) and $57 \pm 2.5\%$ ($t_{(15)} = 2.7$, $P < 0.01$), respectively. In summary, these results show that, in our experiments, the temporal dynamics of neuronal ensemble activity could specify the identity of natural rewards even before a licking cluster started.

Reliability and redundancy in the ensemble classification of sucrose or water

Thus far, we have shown that the information obtained from OFC neuronal ensembles can be used to discriminate between licking for sucrose and water and that the information relating to this discrimination increases with the size of the ensemble. However, we now inquire as to the reliability of this discrim-

ination within an ensemble. Specifically, we ask whether the ensemble will perform better than individual neurons. To address these questions, we reanalyzed our data (± 500 ms relative to the start of the cluster) using the neuronal ensembles that discriminated between sucrose and water in which >10 neurons were simultaneously recorded ($n = 6$ ensembles). From these experiments, individual neurons were randomly removed from the neuronal ensemble, and the performance of the remaining neurons was calculated, until only one neuron was left in the sample. We found that the ensemble performance improved with the number of neurons in each ensemble, suggesting that a reliable and better performance is achieved when the ensemble size increases (Fig. 6D). This of course, does not imply that single neurons cannot discriminate between gustatory stimuli. In fact, the average performance of single neurons was $56.3 \pm 1.2\%$ ($n = 87$), which is significantly greater than chance level (t -test, $t_{(86)} = 5.3$, $P < 0.0001$). Moreover, 60 of the 87 neurons performed above chance level in discriminating between sucrose and water, and the average performance of these neurons was $61.5 \pm 1.2\%$ ($n = 60$). Nevertheless, the ensemble performance was $75.5 \pm 4.5\%$, which is significantly greater than the single unit performance ($n = 6$ ensembles, t -test, $t_{(59)} = -14$, $P < 0.0001$).

Similar results were found when all 16 experiments involving the licking for sucrose and water were analyzed. Under these conditions, the average single unit performance was $54.5 \pm 0.8\%$, and 98 of 152 neurons performed above chance level, averaging $60 \pm 0.8\%$. However, as before, the mean ensemble performance (62.3%) was significantly greater than even the average performance of these 98 cells (t -test, $t_{(97)} = -2.9$, $P < 0.01$).

To address the extent to which an individual neuron contributes to the performance of the ensemble, we calculated the difference in an ensemble's performance with all the neurons minus the ensemble's performance without one neuron. We found that 23% (20/87) of the neurons reduced the ensembles performance when they were removed from the ensemble. On average, the ensemble performance decreased $4.7 \pm 0.72\%$ /neuron, suggesting that the removal of any neuron did not greatly reduce the performance of the whole ensemble. On analyzing all of the experiments, it was found that 31 of 152 neurons reduced the ensemble performance, with the mean reduction of performance being $5.6 \pm 0.72\%$ /neuron. This was also the case even when the best single neuron, that is, the neuron that by itself reached the best performance, was removed from the ensemble (Fig. 6E, gray box). This result indicates the existence of redundant information in the ensemble (distributed coding). Finally, as expected from a redundant system, we found that the performance of the neuronal population slowly decayed when the best single neurons were removed (see Fig. 6E; Narayanan et al. 2005).

Classification of the start and termination of a licking cluster

The responses described in Fig. 5 and Table 1 show that many OFC neurons exhibit several changes in firing rate before, during, or after a cluster. This suggests that the pattern of OFC neuronal activity may contain additional information that represents the cluster start or termination. To test this hypothesis, once again we used the PSTH template algorithm

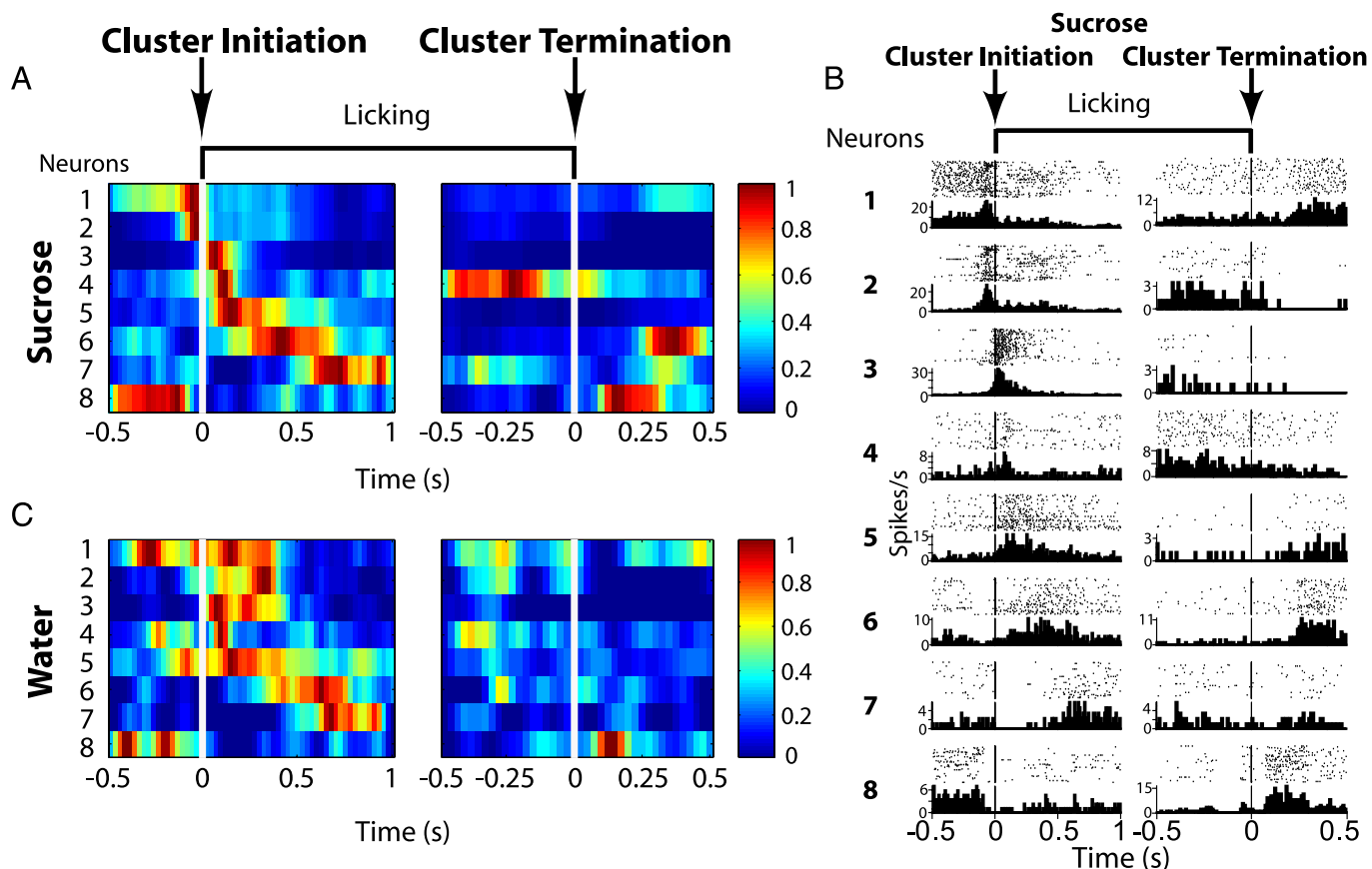


FIG. 5. Ensemble of OFC neurons obtained during ad libitum ingestion of 0.4 M sucrose and water. *A* (sucrose) and *C* (water) are color-coded PSTHs of simultaneously recorded OFC neuronal responses. Times around cluster initiation (*left*) and cluster termination (*right*) are presented. The same row in both panels represents activity of the same neuron and each row is a different neuron (rows 1–8). Normalization was accomplished by dividing smoothed firing rates of PSTHs by their respective maximum firing rate. Red, maximal activity; blue, minimum activity of each single unit. *B*: depiction of standard PSTHs and raster plots of the same neurons plotted in *A*.

analysis described above. Templates of cluster start and termination were made and single trials were classified (Fig. 7*A*). The overall classification performance of the neuronal ensembles was $83 \pm 2.6\%$ ($n = 19$ experiments) correct single trial classifications, when 1 s (± 500 ms) of activity was used to construct the templates (see Fig. 7*B*). The classification performance increased with the number of neurons in the ensemble ($r^2 = 0.4$, $F_{(1,17)} = 11.4$, $P < 0.01$, Fig. 7*C*). These results indicate that ensembles of OFC neurons display a specific activation pattern for the start and termination of a cluster.

We also studied the shortest time in which OFC neuronal ensembles could distinguish between the start and termination of a cluster. It was found that the overall classification performance only decreased to $73 \pm 3\%$ ($P < 0.05$) when 100 ± 50 ms of activity was used to make the templates. These results suggest that, even at these short times, OFC ensemble activation patterns were sufficiently stable and distinct to specify the start and termination of a licking cluster.

As shown for the sucrose versus water discrimination, our analysis revealed that OFC ensemble performance was more reliable than individual units in discriminating cluster start from termination. Although, most of the OFC neurons performed above chance level (mean $70 \pm 1\%$; $n = 112$ of 118 neurons), the ensemble performance was significantly greater ($89.6 \pm 1.8\%$, $n = 9$ ensembles, t -test, $t_{(111)} = -17$, $P < 0.0001$). In addition, by individually removing each neuron

from its ensemble, we found that only 52 of the 118 neurons were able to reduce the ensemble performance, with the average decrease of performance being $1.3 \pm 0.25\%$ /neuron. This indicates that removing any single neuron from the ensemble did not greatly affect the performance of the whole population sampled in each recording session. As before, the ensemble performance slowly decayed even when the best single neuron(s) were removed (Fig. 7, *D* and *E*), indicating that the information was redundant and distributed.

OFC activity predicts the onset of reward (onset of a licking cluster)

The third analysis of the OFC ensemble activity that was performed involved predicting when a freely moving rat will actually lick to begin a cluster. Predictions were obtained by linearly mapping the ensemble activity with the licking behavior (see METHODS). The neuronal ensemble prediction of 13 OFC simultaneously recorded neurons is shown in Fig. 8*A*. In this figure, the times at which the rat licked to start a cluster are indicated in blue, and the neuronal predictions are in red. It is clear that the linear model accurately predicts when the animal licked sucrose to begin a cluster. Also shown are two examples where the linear model yielded false-positive predictions (arrows). To show that the correct predictions of the model were not fortuitous, the lick sequence was shifted 1 s with respect to

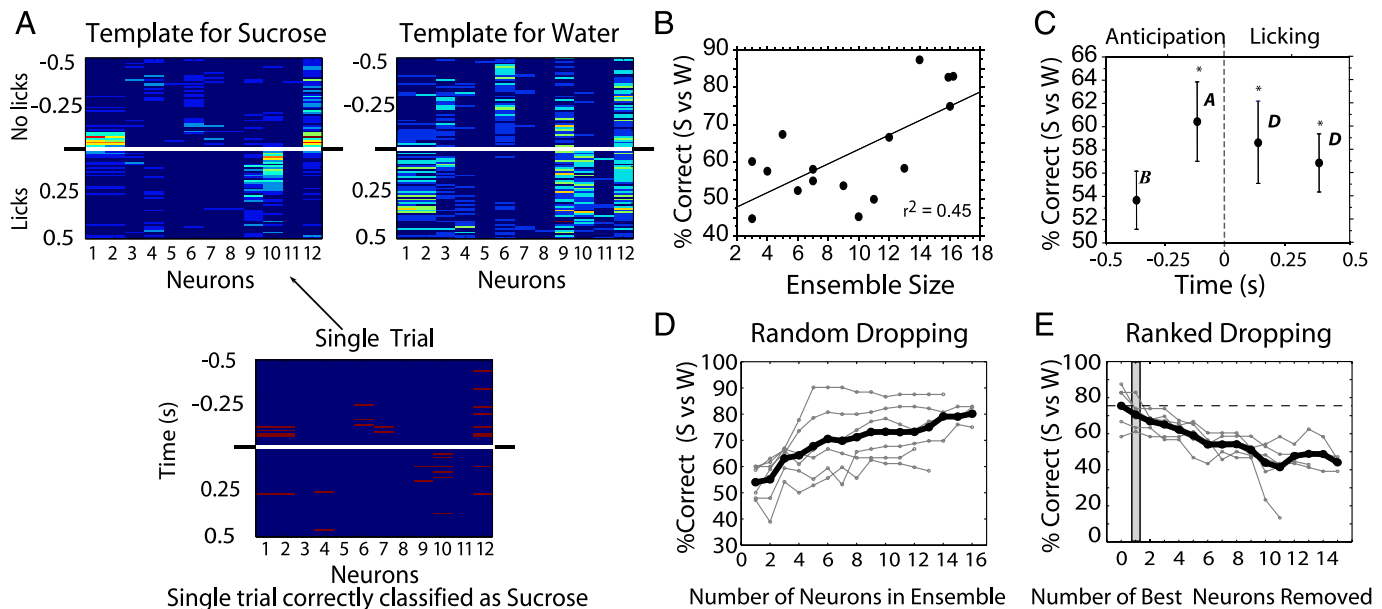


FIG. 6. OFC ensemble activity can distinguish between sucrose and water. *A*: graphical representation of activation patterns or templates of 12 simultaneously recorded OFC neurons obtained while a rat drank 0.4 M sucrose or water. Also shown is a single testing trial (1 individual cluster start for sucrose) that was correctly classified as sucrose. Templates contain mean neuronal activity (counts/bin) of all trials except the 1 that is being classified. *Time 0* corresponds to the 1st lick in a cluster. *B*: correlation between ensemble size (number of neurons) and ensemble classification performance. Circles represent percentage performance of individual OFC ensembles, and each circle depicts different experimental sessions. *C*: graph showing reward classification performance for 4 epochs: “B” baseline, “A” approach, and “D” drinking (each epoch was 240 ms). Data are presented as means \pm SE ($n = 16$ experiments). *D*: random neuron-dropping curves describing classification performance for sucrose or water is shown as a function of the number of neurons in an ensemble. Gray thin lines, individual experiments; bold line, average dropping curve for all experiments. A reliable performance was achieved when there were 9–10 neurons in the ensemble. *E*: rank dropping analysis of the same ensembles plotted in *D*. Note the slow decay in performance when the 1st best neuron of each ensemble was removed (gray box). Dashed line represents average performance of whole ensembles.

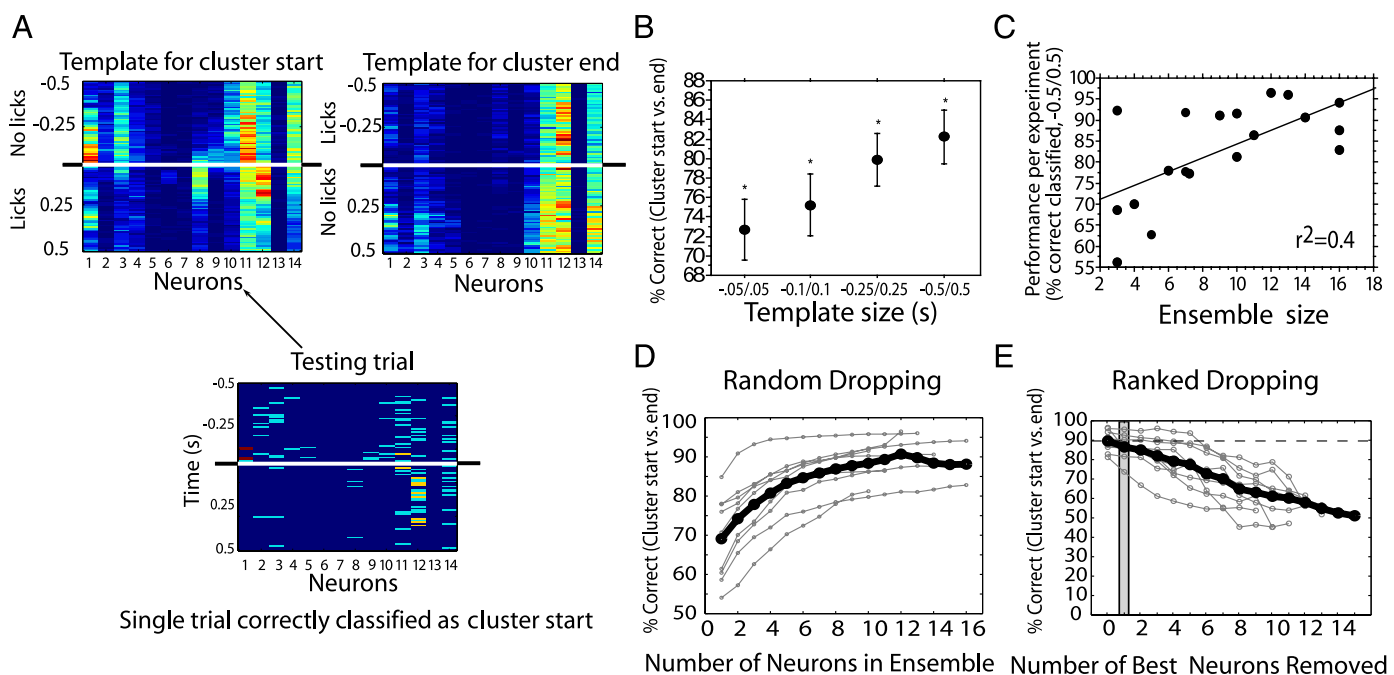


FIG. 7. Classification of cluster start and termination. *A*: graphical representation of activation patterns (or templates) of 14 simultaneously recorded OFC neurons obtained while a rat licked for sucrose that is used to classify cluster start and termination. Also shown is a single testing trial (1 individual cluster start) that was correctly classified. *B*: percentage of correctly classified trials of all the experiments ($n = 19$) using several templates sizes to construct the templates and testing trials. *C*: graph showing a positive correlation between ensemble size and classification performance at the largest tested template (± 500 ms around cluster start or end). Data are presented as means \pm SE ($n = 19$ experiments). *D*: random neuron-dropping curve describing classification performance for start and termination of the cluster (± 500 -ms template). Note that ensemble performance improved with the number of neurons in each ensemble. *E*: rank dropping analysis of the same ensembles plotted in *D*. Observe that the ensemble performance gradually decayed when the 1st best neuron of each ensemble was removed (gray box). Same conventions as in Fig. 6, *D* and *E*.

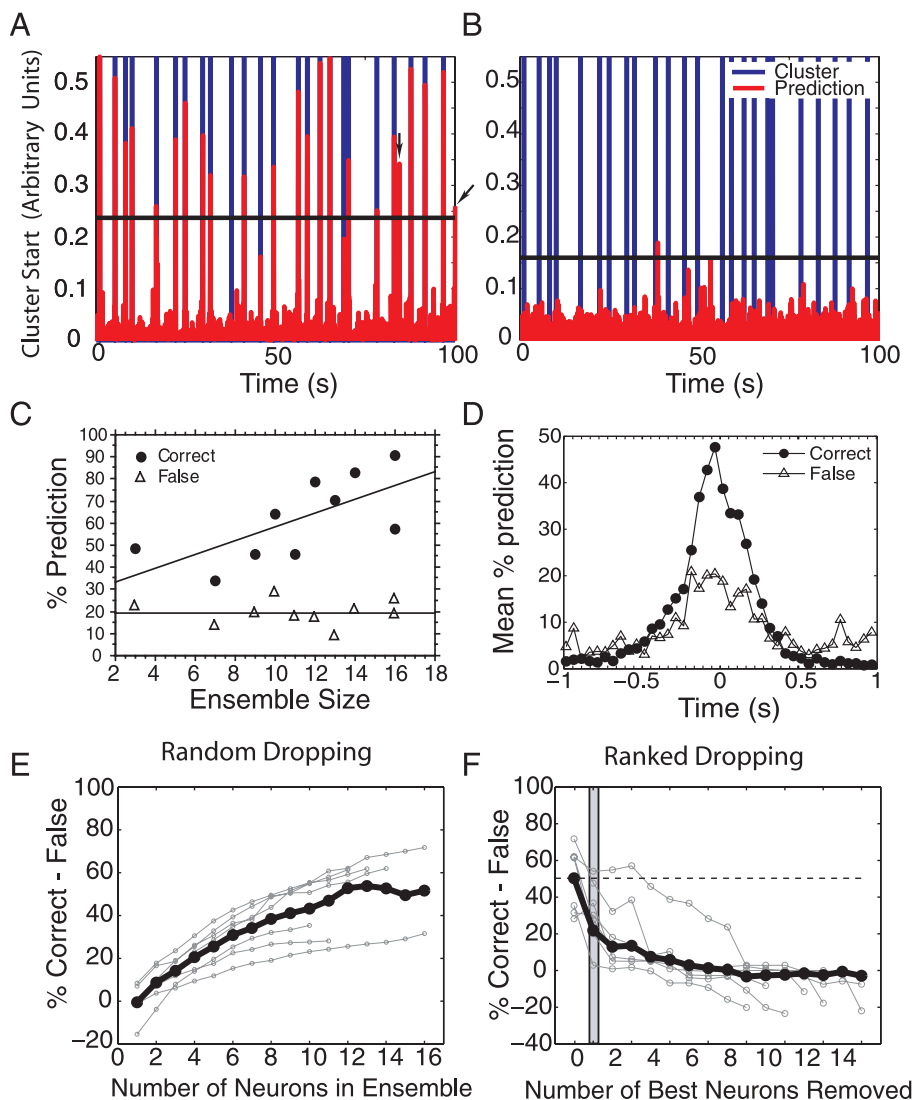


FIG. 8. OFC neuronal ensemble predicts cluster initiation. *A*: comparison of observed initiation of licking clusters (blue) and ensemble predictions (red) across a 100-s window. Threshold level is depicted as a black line. This ensemble was composed of 13 simultaneously recorded neurons. False positives (arrows) are classified as ensemble predictions above threshold level that do not coincide with onset of licking cluster. *B*: control study showing that when licking behavior was shifted 1 s ahead with respect to neuronal activity, there was a very significant decrease in ensemble predictions. Conventions are the same as in *A*. *C*: plot showing quality of model predictions as a function of ensemble size for both true positives (circles) and false positives (triangles). *D*: perievent analysis of ensemble performance for onset of licking cluster. Each point is the average performance, in 50-ms bins, across experiments ($n = 10$) for both true positives (circles) and false positives (triangles) cases. Note that the ensemble performance increased in anticipation of cluster start and rapidly decayed in the next 200 ms. *E*: random dropping curve. *F*: ranked dropping curve for ensemble accuracy for predicting cluster initiation. Same conventions as in Fig. 6, *D* and *E*.

the neuronal activity. This shift reduced the predictive accuracy of the model essentially to zero (Fig. 8*B*), thereby showing that the linear model can accurately predict the behavior of a freely moving rat. The performance of individual experiments is shown in Fig. 8*C*.

Further analysis of the OFC activity showed that the model achieved its maximum performance when the neuronal activity was between 250 ms before and 250 ms after the cluster start ($t = 0$ s). Under this condition, the model correctly detected $62 \pm 6\%$ of the time ($n = 10$ experiments) when the animal licked to begin a cluster (Fig. 8*C*). As expected, the greater the number of neurons in the ensemble, the greater the models' predictive value ($r^2 = 0.46$, $F_{(1,8)} = 6.9$, $P < 0.05$). This analysis revealed that the number of false positives predicted by the model ($19 \pm 2\%$) was independent of the ensemble size ($r^2 = 0.01$, $P > 0.05$). Also seen in the figure is that some of the smaller ensembles gave better predictions than the larger ones. This indicates that some single neurons provide more precise information regarding the start of a cluster.

The temporal dynamics of the ensembles' prediction were revealed by the perievent analysis of the ensemble performance. This analysis showed a 150-ms ramping increase in the ensembles' performance that peaked 50 ms before the start of

a cluster and decayed over the subsequent 200 ms (Fig. 8*D*). In summary, these results suggest that the OFC contained information relevant to when the animal intended to initiate a cluster of sucrose.

Accuracy in single units and ensemble performance

The accuracy for detecting the onset of a licking cluster was calculated as the difference between correct and false positive predictions, and thus it represents the certainty with which we can detect a cluster start using the OFC neuronal activity. Therefore an accuracy of 0% indicates the inability to detect the onset of a licking cluster. By randomly removing neurons, we found that the accuracy of the linear model improved with the number of neurons in the ensemble (Fig. 8*E*). The average accuracy in the neuronal ensembles was $50 \pm 6.7\%$ ($n = 7$ ensembles), which is significantly greater than the mean accuracy of single units ($4.0 \pm 1.6\%$). In fact, only 39 of 92 single neurons had an accuracy value $>0\%$, (i.e., the linear model detected more true than false positives) and the average accuracy of these 39 neurons was $16 \pm 1.6\%$. Thus single neurons were significantly less accurate than the ensembles (t -test, $t_{(38)} = -13$, $P < 0.0001$). In addition, 55 of 92 neurons

reduced the accuracy of the neuronal ensemble when an individual neuron, was removed from the ensemble. Although the average drop in performance was low ($4.6 \pm 1.5\%$ /neuron), selected neurons ($n = 4$) greatly reduced the accuracy of the ensemble, indicating that some single neurons greatly contributed more to the ensemble performance. To this point, in four of seven experiments, a rapid fall in performance was observed when the best single neuron of each ensemble was removed (Fig. 8F). In summary, this result indicates that during detection of a freely moving rat initiating a licking cluster, some highly specific neurons appear to contribute more to the accuracy of the ensembles than other neurons, but the ensemble activity was always more accurate than any single neuron to detect the onset of a licking cluster.

Cluster criteria

There have been several discussions in the literature regarding whether a cluster should be defined by pauses of 0.5 or 1 s (Spector et al. 1998). To address this issue, we analyzed the ensemble's ability to predict when a rat will lick to begin a cluster using ICIs of 0.5 and 1 s. We found that with an ICI = 1 s, the overall mean ensembles' performance for the prediction of cluster start was reduced to $47 \pm 7\%$ (true positives) and $19 \pm 4\%$ (false positives) relative to performance found with a 0.5-s cluster definition (62 ± 6 and $19 \pm 2\%$ true and false positives, respectively). This result indicates that the use of a pause of 0.5 s to define a cluster (Davis and Smith 1992; Taha and Fields 2005) gives better correlations between the behavior and neuronal activity in OFC.

In contrast with the high quality of predictions of the first lick in a cluster, the last lick in a cluster was poorly predicted, reaching average performance of $45 \pm 11\%$ in only 2 of 10 experiments. Interestingly, the neuronal activity that best correlated with the end of the cluster was precisely localized between 150 and 400 ms after the last lick (recall that median duration ICI is 1.2 s). This suggests that additional or larger ensembles containing such neurons should allow an accurate prediction of when the animal will stop drinking and therefore when the cluster will terminate. It is also important to mention that when clusters were defined following the ICI = 1 s criteria, the detection of the cluster end decreased to 6%. This result once again indicates that the OFC ensemble activity describes better the clusters when an ICI = 0.5 s criteria is used to define the clusters.

Histology

The histological results seen in supplementary Figs. S1 and S2 confirm that the cannulae were implanted in the OFC and that the electrodes recorded neuronal activity from the lateral and dorsolateral part of the OFC.

DISCUSSION

The classification of rhythmic licking into clusters (Davis and Smith 1992) has been very useful for the study of ingestive behavior and the characterization of compounds that alter the duration or number of the clusters (Hartfield et al. 2003; Higgs and Cooper 2000; Higgs et al. 2003; Stratford et al. 1995). This is because the cluster duration increases with the tastants' palatability (Taha and Fields 2005). Here, for the first time, we

showed that neurons in the OFC, an area of the brain that is responsive to the hedonic nature of food (de Araujo et al. 2003), fire during different parts of the cluster. Analysis of OFC ensemble activity obtained from freely licking animals revealed an accurate prediction of when an animal will first lick to begin a cluster (Fig. 8). Moreover, the information in the ensembles is sufficient to distinguish between sucrose and water after one or two licks and when it is anticipated (Fig. 6). This activity suggests a new role for the OFC in the ingestion of liquid rewards.

Role of the OFC in tongue movements and reward

One reason that we measured the activity of rat OFC neurons is that this cortical area is important in voluntary licking behavior. Specifically, electrical stimulation of the OFC was shown to induce rhythmical jaw or tongue movements (Neafsey et al. 1986), and ablation of the OFC results in deficits in tongue protrusion and in the use of the mouth and tongue (Whishaw and Kolb 1983). Another reason is the role of the OFC in reward. Animals are motivated to lick for water because they are seeking a reward. In this regard, licking is inextricably associated with reward. The cortical area, in which we recorded, the lateral OFC, is connected with reward-related structures such as the ventral tegmental area and nucleus accumbens. In addition, the OFC receives information from all sensory modalities, including taste (Cavada et al. 2000). It follows that the lateral OFC is in an excellent position to monitor the consumption of palatable and rewarding solutions. Indeed, functional MRI studies in humans have found that activity in the OFC is correlated with pleasantness of food (Kringelbach et al. 2003). Because a licking cluster may also be an index of taste palatability, it may be expected that OFC activity tracks the initiation and progress of a licking cluster.

Effects OFC inactivation on the microstructure of licking

Inactivating the OFC produces a fragmented pattern of licking as evidenced by the increase in number of clusters and the decrease in cluster duration (Fig. 1C). These results suggest that OFC activity may be necessary to prevent an excessive number of interruptions during licking, thus rationalizing why larger-sized clusters are observed under normal conditions. The absence of changes in the licking rate and in the number of licks argues against a gross sensory-motor problem involving tongue movements. However, on average, for each lick, these animals ingested about a 33% smaller fluid volume than controls. This reduction likely arises as a consequence of shorter tongue protrusions as occurs in OFC-lesioned rats (Whishaw and Kolb 1983). To this point, almost identical changes in the microstructure of licking were found in rats chronically treated with amphetamine (Wolgin and Jakubow 2003), a drug that was found to decrease dendritic spines in pyramidal neurons in the lateral OFC (Crombag et al. 2005). In summary, we found that inactivation of the OFC affects the neural circuitry related to organize licking clusters, suggesting that the OFC has a role in anticipating and modulating the clusters of rewarding gustatory stimuli.

In addition, inactivation of the OFC could also have reduced the palatability of sucrose. This is because this brain region encodes the reward value of foods and because its inactivation

will shorten the cluster duration, which is used as an index of palatability (Davis and Smith 1992). Although our results are consistent with this view, further experiments using additional measures of palatability and different tastants are required to better understand the role the OFC plays in gustatory palatability.

Oscillatory OFC neurons

Many OFC neurons have been found to be multimodal and therefore may participate in functions other than those we described (Elliott et al. 2000; Rolls 2000). Nevertheless, our electrophysiological recordings revealed the presence of OFC neurons whose firing pattern tracks the licking frequency (~6–7 Hz). When the neuronal responses to both water and sucrose exhibited both the same magnitude and phase, it is probable such neurons convey oromotor and somatosensory information (Fig. 4A). When the responses to water and sucrose differ in magnitude (Fig. 4, C and D) and/or phase of the licking cycle (Fig. 4C), they likely integrate somatosensory and gustatory information. This interpretation is supported by a behavioral study that found similar licking rates and force per lick for water and glucose solutions (Vrtunski et al. 1977). These types of oscillatory activity in the OFC have not previously been reported. Licking-related activity has not been reported in recordings obtained from monkey OFC (Thorpe et al. 1983; Tremblay and Schultz 2000), although neurons responsive to oral movements were recently found in area 12 of the OFC (Ifuku et al. 2003). Licking-related activity has been found in several brain stem and cerebral regions including the gustatory cortex (Katz et al. 2001; Yamamoto et al. 1989b), striatum (Nakamura et al. 1990), amygdala (Kaku 1984), lateral hypothalamus (Yamamoto et al. 1989a), and lateral reticular formation (Travers et al. 1997). In none of these structures have neuronal responses related to cluster initiation or termination been identified, although they may be present. The identification of OFC evoked activity that occurs during specific parts of a licking cluster suggests that, among its many other functions, the OFC may be part of the neural circuitry that organizes the microstructure of licking in ad libitum access to liquid rewards.

Activity in and around licking clusters

Our data show that there are different types of information in the OFC activity related to licking clusters. Specifically, OFC ensemble activity can be used to predict when a behaving rat actually licks to begin a cluster (Fig. 8), information about the start and end of a cluster (Fig. 7), and information that can discriminate between two natural rewards (water and sucrose; Fig. 6). That OFC neurons distinguish among rewarding stimuli has previously been shown (Hikosaka and Watanabe 2000; Thorpe et al. 1983; Tremblay and Schultz 1999). However, it has not been previously shown in freely licking animals that the ensemble activity can be used to discriminate between water and sucrose 250 ms before the cluster starts; i.e., when the animal anticipates the receipt of the sensory and/or the reward that it expects (Fig. 6). This suggests that OFC neurons may provide information about the upcoming tastant even when no explicit instructions (predictive cues in behavioral tasks) are present. In this circumstance only, the volition of the

animal determines when the solution will be ingested. In summary, OFC ensembles contain several “types” of neurons (Table 1) that participate, to various extents, in contributing to the ensemble’s ability to distinguish between sucrose and water and predict when an animal will lick. This richness of information may be important to monitor and influence the clusters during ingestion of liquid rewards.

One physiologically relevant aspect of this study was discovering that OFC activity around a licking cluster can be used to distinguish between sucrose and water in one or two licks (i.e., <250 ms; Fig. 6C). These data are consistent with behavioral studies by Halpern and Tapper (1971), which determined that trained rats could identify a tastant and its reward value during this short time period. Perhaps the more important result was that OFC activity could distinguish between sucrose and water even before the animal tasted them (Fig. 5C). We hypothesize that this will only occur under conditions in which the type of solution is predictable. We also note that not all the OFC ensembles could discriminate between water and sucrose. This suggests that some OFC neurons may form functional ensembles that anticipate and discriminate between gustatory stimuli, whereas others may convey other types of information.

This study also shows that the OFC ensemble activity is a good predictor of the reward onset (onset of a licking cluster; Fig. 7). This suggests that OFC ensemble activity is more closely related to the initiation of goal-directed behavior and may be related to the affinity of OFC neurons to respond before the reward or to external stimuli that predict reward delivery (Schoenbaum et al. 1998).

The OFC is reciprocally connected to areas that modulate licking; the primary gustatory cortex (GC) (Yamamoto et al. 1989b) and the amygdala (AM) (Kaku 1984). The GC has been shown to provide an accurate representation of taste quality and quantity (Plata-Salaman et al. 1996; Scott et al. 1991), and the AM and the OFC underlie much of the neural processing involved in the evaluation of the hedonic properties of food and the motivation to eat or drink (Hinton et al. 2004; Pickens et al. 2003; Schoenbaum et al. 2003; Tremblay and Schultz 1999; Yan and Scott 1996). Our identification of licking-related OFC activity, that OFC ensembles can be used to predict when a rat will begin to lick for sucrose, and that they contain information pertaining to the identity of the taste solution are consistent with its roles in motivation and feeding. The interaction of the OFC with other reward-related areas may be important for the modulation of licking clusters. A recent study of recordings from neurons of the nucleus accumbens (NAcc; which receives OFC projections) from rats performing a sucrose discrimination paradigm found that a majority of them were inhibited before they initiated a licking cluster, whereas others that occurred in the cluster responded in a manner that was related to the palatability of sucrose (Taha and Fields 2005). These results suggest that the interaction of the OFC and NAcc is likely to be important to better understand the initiation and modulation of licking clusters.

In conclusion, our results show that ensembles of multimodal OFC neurons can monitor the ingestion of natural rewards by tracking the onset of a licking cluster as well anticipating and identifying the ingestion of natural rewards.

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