THE MEDIAL FRONTAL CORTEX GENERATES RHYTHMIC ACTIVITY THAT ENCODES REWARD VALUE

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Abstract

Rodents lick to consume fluids. The reward value of ingested fluids is likely to be encoded by neuronal activity entrained to the lick cycle. Here, we investigated relationships between licking and reward signaling by the medial frontal cortex [MFC], a key cortical region for reward-guided learning and decision-making. Multi-electrode recordings of spike activity and field potentials were made in male rats as they performed an incentive contrast licking task. The rats received access to higher and lower value sucrose rewards over alternating 30 sec periods. They learned to lick persistently when the higher value reward was available and to suppress licking when the lower value reward was available. Spectral analysis of spikes and fields revealed evidence for reward value being encoded by the strength of phase locking of 4-12 Hz rhythmic activity to the rats' lick cycle. Recordings during the initial acquisition of the task found that the strength of phase locking to the lick cycle was strengthened with experience. A modification of the task, with a temporal gap of 2 sec added between reward deliveries, showed that the rhythmic signals persisted during periods of dry licking, a finding that suggests the MFC encodes the value of the currently available reward. Finally, we found that reversible inactivations of the MFC in the opposite hemisphere eliminated the encoding of reward information. Together, our findings establish that 4-12 Hz rhythmic activity is generated by neurons in the MFC to encode the value of consumed rewards.

Significance Statement

The cellular and behavioral mechanisms of reward signaling by the medial frontal cortex [MFC] have not been resolved. We report evidence for a dynamic encoding of reward value by neuronal activity in the MFC that is synchronized with ongoing consummatory actions. Previous studies of MFC reward signaling have inferred value coding upon temporally sustained activity during the period of reward consumption. Our findings suggest that MFC activity is temporally sustained due to the consumption of the rewarding fluids, and not necessarily the abstract properties of the rewarding fluid. Two other major findings were that the MFC reward signals persist beyond the period of fluid delivery and are generated by neurons within the MFC.

Introduction

Reward-related neuronal activity is commonly found in the medial frontal cortex [MFC] of humans (Glascher et al., 2009; Levy and Glimcher, 2011), primates (Watanabe, 1996; Shidara and Richmond, 2002; Roesch and Olson, 2004; Amiez et al., 2006; Padoa-Schiappa and Assad, 2006; Hayden et al., 2009; Luk and Wallis, 2009; Bouret and Richmond, 2010; Cai and Padoa-Schioppa, 2012), and rodents (Petyko et al., 2009; Horst and Laubach, 2012, 2013; Donnelly et al., 2014; Petyko et al., 2015). However, the behavioral determinants of these signals are not understood. In neurophysiological studies in experimental animals, rewards are typically given as liquids (Apicella et al., 1991; Carelli and Deadwyler, 1994) to avoid issues with chewing and grinding foods. A fixed cycle of specific behaviors (jaw opening, tongue protrusion and retraction, jaw closing, swallowing) underlies the processing of liquid rewards. These behaviors should have a major impact on reward signaling by the MFC.

Indeed, Horst and Laubach (2012) reported that MFC activity is sharply modulated when thirsty rats lick to consume water rewards in a MFC-dependent working memory task (Horst and Laubach, 2009). By modifying the task to delay the delivery of water on some trials, a subpopulation of MFC neurons was revealed that were selectively activated by the initiation of licking (Horst and Laubach, 2013). These changes in spike activity are accompanied by prominent fluctuations of MFC local field potentials [LFPs], specifically near the rats' licking frequency (5-7 Hz). These signals were most prevalent in the most rostral MFC. A subsequent study found that reversible inactivation of this rostral part of the MFC dramatically reduces the duration of licking bouts (Parent et al., 2015a), similar to how inactivation of the more caudal

MFC leads to excessive premature responding in tasks that require actions to be sustained over delay periods (e.g., Narayanan et al., 2006). These studies suggest that the rostral MFC is specialized for the value-guided control of consummatory behavior.

The goal of the present study was to determine if licking-related neuronal activity in the rostral MFC is sensitive to the reward value of ingested fluids. To examine this issue, we used a simple take-it-or-leave decision-making task, called the Shifting Values Licking Task (Parent et al., 2015a,b), to study reward signaling in relation to ongoing consummatory actions. Rats lick on a spout to receive liquid sucrose rewards and the concentration of sucrose alternates between a higher (better) and lower (worse) option every 30 sec. After only a few days of training, the rats learn to persistently lick when the better option is available and to suppress licking when the worse option is available. Bilateral reversible inactivations of the rostral MFC impair performance in this task (Parent et al., 2015a), resulting in temporally fragmented licking (dramatic reductions in the duration of licking bouts). Opposite effects are found after intra-MFC infusions of drugs that are known to enhance neuronal excitability near the licking frequency, such as the M-channel blocker XE-991 (Hu et al., 2002), and the “hunger hormone” ghrelin (Parent et al., 2015b).

To examine how the rostral MFC encodes relative reward values and controls value-guided consumption, we recorded spike activity and local field potentials [LFP] as rats performed the Shifting Values Licking Task. We found that neuronal activity in the MFC encodes reward value by showing stronger entrainment to the lick cycle when animals ingest high-value sucrose rewards. These signals developed with experience, persisted beyond periods of fluid

delivery, and depended on MFC neurons. Together, our findings suggest that MFC neurons track engagement in consummatory behavior and encode the value of rewarding foods and fluids.

Materials and Methods

Procedures were approved by the Animal Use and Care Committees at the John B. Pierce Laboratory (where some of the experiments were conducted) and American University and conformed to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were taken to minimize the number of animals used and to reduce pain and suffering.

Experimental Design

Animals

Male Long Evans rats weighing between 300 and 325 g were purchased from Harlan or Charles River. Rats were given one week to acclimate with daily handling prior to behavioral training or surgery and were then kept with regulated access to food to maintain 90% of their free-freeding body weight. They were given ~18g of standard rat chow each day in the evenings following experiments. Rats were single-housed in their home cages in a 12h light/dark cycle colony room, with experiments occurring during the light cycle. A total of 11 rats had a microwire array implanted into medial frontal cortex. Some rats additionally had a drug cannula implanted into the opposite hemisphere using the same stereotaxic coordinates. Arrays were made by Tucker-Davis Technologies and consisted of 16 blunt-cut 50-μm tungsten wires,

insulated with Formvar, separated by 250 μm within each row and 500 μm between rows. In vitro impedances for the microwires were ~150 kΩ.

Surgeries

Animals had full access to food and water in the days prior to surgery. Stereotaxic surgery was performed using standard methods (e.g., Narayanan and Laubach, 2006). Briefly, animals were lightly anesthetized with isoflurane (2.5% for ~2 min), and were then injected intraperitoneally with ketamine (100mg/kg) and either xylazine (10 mg/kg) or dexdomitor (0.25mg/kg) to maintain a surgical plane of anesthesia. Craniotomies were made above the implant location. Microwire arrays were placed into the medial frontal cortex (coordinates from bregma: AP: +3.2 mm; ML: ± 1.0 mm; DV: -2.2 mm from the surface of the brain, at a 12° posterior angle). Four skull screws were placed along the edges of the skull and a ground wire was secured in the intracranial space above the posterior cerebral cortex. Electrode arrays were connected to a headstage cable and modified Plexon preamplifier during surgery and recordings were made to assess neural activity during array placement. Drug cannulas, 26-gauge PEEK (Plastics One), were implanted prior to the microwire arrays using similar procedures. Craniotomies were sealed using cyanocrylate (Slo-Zap) and an accelerator (Zip Kicker), and methyl methacrylate dental cement (AM Systems) was applied and affixed to the skull via the skull screws. Animals were given a reversal agent for either xylazine (yohimbine, 2mg/ml) or dexdomitor (Antisedan, s.c. 0.25 mg/ml) and Carprofen (5 mg/kg, s.c.) was administered for postoperative analgesia. Animals recovered from surgery in their home cages

for at least one week with full food and water, and were weighed and handled daily.

Behavioral Apparatus

Rats were trained in operant chambers housed within a sound-attenuating external chamber (Med Associates, St. Albans, VT). Operant chambers contained a custom-made drinking spout that was connected to multiple fluid lines allowing for multiple fluids to be consumed at the same location. The spout was centered on one side of the operant chamber wall at a height of 5 to 6.5cm from the chamber floor. Tygon tubing connected to the back of the drinking spout would administer the fluid from a 60cc syringe hooked up to a PHM-100 pump (Med Associates). A “light-pipe” lickometer (Med Associates) detected licks via an LED photobeam, and each lick triggered the pump to deliver roughly 0.025 ml per 0.5 sec. Behavioral protocols were run though Med-PC version IV (Med Associates), and behavioral data was sent via TTL pulses from the Med-PC software to the Plexon recording system.

Continuous-Access Shifting Values Licking Task

The operant licking task used here is similar to that previously described in Parent et al. (2015a,b). Briefly, rats were placed in the operant chamber for 30 min, where they were solely required to lick at the drinking spout to obtain a liquid sucrose reward. Licks activated the syringe pumps to deliver liquid sucrose over 0.5 sec. Every 30 sec, the reward alternated between a high concentration (20% weight per volume) and low concentration (2-4% wt/vol) of sucrose. The animal’s licking behavior was constantly monitored.

Instrumental Shifting Values Licking Task

The operant licking task used above was modified slightly to allow for assessment of reinforced versus non-reinforced licks. A 2-sec inter-pump interval was included between each pump activation. In other words, the rat would lick to activate a liquid sucrose reward for 0.5 sec, and then once the pump stopped delivering fluid, no reward was delivered again for 2 sec. The next lick after the 2 sec interval would initiate the next pump activation. Licks during the 2 sec inter-pump interval were *instrumental*.

Multi-Electrode Recordings

Electrophysiological recordings were made using a Plexon Multichannel Acquisition Processor (MAP; Plexon; Dallas, TX). Local field potentials were sampled on all electrodes and recorded continuously throughout the behavioral testing sessions using the Plexon system via National Instruments A/D card (PCI-DIO-32HS). The sampling rate was 1 kHz. The head-stage filters (Plexon) were at 0.5 Hz and 5.9 kHz. Electrodes with unstable signals or prominent peaks at 60 Hz in plots of power spectral density were excluded from quantitative analysis.

Reversible Inactivation

Animals were tested with muscimol infusions in one hemisphere and recordings of neural activity in the opposite hemisphere. For control sessions, phosphate-buffered saline (PBS) was infused into MFC. The next day, muscimol (Sigma-Aldrich, St Louis, MO) was infused at 0.1 μg/μl.

Infusions were performed by inserting a 33-gauge injector into the guide cannula, and 1.0 µl of fluid was delivered at a rate of 15 µl per h (0.25 µl per min) with a syringe infusion pump (KDS Scientific, Holliston, MA). The injector was connected to a 10 µl Hamilton syringe via 0.38 mm diameter polyethylene tubing. After infusion was finished, the injector was left in place for at least 4 min to allow for diffusion of the fluid. The injector was slowly removed and the headstage cable was subsequently plugged into the animal's implant. Rats were tested in the instrumental Shifting Values Licking Task 1 hour after the PBS or muscimol infusions. Recordings were made the day following the infusion session without any manipulation to verify recovery from the inactivation session.

Histology

After all experiments were completed, rats were deeply anesthetized via an intraperitoneal injection of Euthasol (100mg/kg) and then transcardially perfused using 4% paraformaldehyde in phosphate-buffered saline. Brains were cryoprotected with a 20% sucrose and 10% glycerol mixture and then sectioned horizontally on a freezing microtome. The slices were mounted on gelatin-subbed slides and stained for Nissl substance with thionin.

Statistical Analysis

Software and Testing

All data were analyzed using GNU Octave (https://www.gnu.org/software/octave/), Python (Anaconda distribution: https://www.continuum.io/), and R (https://www.r-project.org/).

Analyses were run as Jupyter notebooks (http://jupyter.org/). Statistical testing was performed using standard (base) packages for R. Standard non-parametric tests and paired t-tests were used throughout the study. Repeated-measures ANOVA (with the error term due to subject) were used to compare data across training sessions (Figure 7), reinforced versus non-reinforced licks (Figure 9), and PBS versus muscimol (Figure 10). Computer code used in this study is available upon request from the corresponding author.

Data Analysis: Local Field Potentials

Electrophysiological data were first briefly assessed in NeuroExplorer (http://www.neuroexplorer.com/). Subsequent processing was done using signal processing routines in GNU Octave. Analysis of LFP data was carried out using the EEGlab toolbox (http://sccn.ucsd.edu/EEGlab/) (Event-Related Spectral Power and Inter-Trial Coherence) and Neurospec 2.0 (http://www.neurospec.org/) (spike-lick and spike-field coherence). Circular statistics were calculated using the *circular* library for R (Agostinelli and Lund, 2013). Graphical plots of data were made using the *matplotlib* and *seaborn* library for Python. Analyses were typically conducted in Jupyter notebooks, and interactions between Python, R, and Octave were implemented using the *rpy2* and *oct2py* libraries for Python.

To measure the amplitude and phase of LFP in the frequency range of licking, LFPs were bandpass-filtered using EEGlab's *eegfilt* function, with a fir1 filter (Widmann & Schröger, 2012), centered at the rat's licking frequency (licking frequency $\pm$ inter-quartile range; typically around 4 to 9 Hz), and were subsequently z-scored. Analyses were performed with a pre/post window of

2 sec, and the Hilbert transform was used to obtain the amplitude and phase of the LFP.

To measure the consistency of LFP phase angles in relation to licking, 500 licks were randomly chosen from one session from each rat along with 500 random time points that were chosen based on shuffling the inter-lick intervals from all licks in the rat's session. After creating peri-event matrices from filtered and z-scored LFP data, the Hilbert transform was applied to obtain the phase angle and amplitude for each electrode, and analyzed with routines from the *circular* library for R. *rho.circular was used* to obtain mean resultant vector length. *mean.circular* was used to obtain average phase. The *rao.spacing.test* function was used to obtain Rao's statistic and corresponding p-value, which indicates if phase angles were uniformly distributed.

For time-frequency analysis (ERSP and ITC), LFPs were preprocessed using EEGlab's *eegfilt* function with a fir1 filter and bandpass filtered from 0 to 100 Hz. For group summaries, ERSP and ITC matrices were z-scored for that given rat after bandpass filtering the data. Peri-lick matrices were then formed by using a pre/post window of 2 sec on each side, and the *newtimef* function from the EEGlab toolbox was used to generate the time-frequency matrices up to 30 Hz. Group summaries for ERSP and ITC were performed by obtaining the peak ITC value within a time window of ±2 interlick intervals (typically ~±375 ms) around licking, and obtaining the peak ERSP value within that same window. Each electrode's peak ERSP and ITC value for each type of lick (high-value or low-value lick) were used in the ANOVAs for group summaries. Finally, a "value index" was calculated to assess differences in ERSP and ITC measures associated with consumption of the higher and lower value rewards, i.e., $(ITC_{Hi} - ITC_{Lo})/ITC_{Hi}$.

Shuffling methods were used to compare ERSP and ITC values for observed and shuffled

licks (obtained by calculating inter-lick intervals, shuffling their trial order, and adding the intervals to the first lick in each behavioral session). This gave a set of surrogate licks with random timing unrelated to the animal's behavior. Subsets of 50 licks and shuffled events were randomly chosen from each behavioral session and ERSP and ITC statistics were calculated for the subsets of observed and shuffled data.

To assess the stability of the ERSP and ITC measures over the test sessions, the data sets were broken into 10 blocks with equal numbers of licks or equal amounts of time into the session. Peak ERSP and ITC values were calculated for frequencies between 4 and 12 Hz and within two inter-lick intervals around each lick. Summaries for each rat used grand average LFPs (z-scored). Plots of peak ERSP and ITC over the two types of blocks (licks, time) revealed no consistent cross-session effects, e.g., due to satiety (de Araujo et al., 2006; Bouret and Richmond, 2010), for both measures of neuronal activity (see Figure 6).

Data Analysis: Spike Activity

Exploratory analysis of on-line identified single units found that spike probabilities at the times of the licks were below 0.1 for all single units recorded in the task. Therefore, we used multi-unit activity [MUA] to relate spike activity to the animals' lick cycles and related LFP signals. MUA was identified using the Plexon Offline Sorter v. 4.3 (Plexon, Dallas, TX). All recorded spike waveforms were thresholded (±2.7 times the standard deviation for the collection of waveforms) and "automatic artifact invalidation" was applied. Then, using routines in NeuroExplorer v. 5 (Nex Technologies, Madison, AL), we measured spike probabilities for all recorded MUAs around

the higher and lower values licks, using 0.001 sec bins. Spike probabilities were compared for the two lick values using a paired t-test (in R). To measure Spike-Field Coherence [SFC], we also used routines (sp2a_m) from Neurospec 2.0 (*http://www.neurospec.org/*), and analyzed bandpass filtered LFP (licking frequency ± inter-quartile range) and the following parameters: Segment power = 10 (1024 points, frequency resolution: 0.977 Hz), Hanning filtering with 50% tapering, and line noise removal for the LFPs at 60 Hz. To measure Spike-Lick Coherence [SLC], we used routines (sp2_m1.m) from Neurospec 2.0. The following parameters were used: Segment power = 12 (4096 points, frequency resolution: 0.244 Hz) and Hanning filtering with 50% tapering.

Validation of cross-hemispheric connectivity using retrograde tracers

The methods for stereotaxic surgery that are described above were used to make injections of Cholera Toxin subunit B in 5 rats to validate cross-hemispheric connections within the rostral MFC, which have not been extensively studied in previous anatomical studies on the most rostral part of this cortical region (cf. Gabbott et al., 2003; Hoover and Vertes, 2007). The CTB had an Alexa Fluor 488 reporter from Molecular Probes and was injected at a 1% concentration and 400 nl volume. A 10 μl glass Hamilton syringe and Narishige motorized microinjector (IMS-10) was used. 10 min was allowed for diffusion after each injection. Brains were extracted using the methods described above for euthanasia and perfusion. Cortical slices were cut in the frontal plane using a freezing microtome and were imaged on fluorescent microscope (BX-51-F, Tritech Research, Los Angeles, CA) using an R1 camera and Ocular software from Qimaging (Surrey, BC).

Results

Multi-electrode recordings in the Shifting Values Licking Task

To investigate the role of the frontal cortex in reward-related consummatory behaviors, we assessed licking behavior in rats while performing simultaneous recordings in the rostral MFC. We trained rats in the Shifting Values Licking Task (Parent et al., 2015a), in which they licked at a drinking spout to receive 0.025 ml of a liquid sucrose reward (Figure 1A). The reward value of the fluid switched between higher (20% sucrose wt/vol) and lower (2 or 4%) levels every 30 sec. Experienced rats (>3 training sessions) licked more for the high-value reward compared to the low-value reward (Figure 1B; paired t-test between high-value and low-value licks: $t_{(8)} = 4.29$, $p = 0.0026$).

Eleven rats were implanted with multi-electrode arrays (Figure 2A). The placement of electrodes is shown in Figure 2B. All electrodes were placed in the medial agranular and prelimbic areas. In four of the rats, a drug cannula was also implanted in the opposite hemisphere using the same stereotaxic coordinates. These animals were used to examine effects of reversible inactivation of the MFC without shutting down local neuronal activity. To confirm that cross-hemispheric connections exist within the rostral MFC region, 5 rats were injected with Cholera Toxin subunit B in the region where the neuronal recordings and reversible inactivations were made (Figure 2C).

Quantification of lick-entrained rhythmic activity

We recorded 159 local field potentials [LFPs] from the MFC in 11 rats as they ingested

liquid sucrose in the Shifting Values Licking Task. An example of licking-related fluctuations in the LFPs is shown in Figure 3A. To measure entrainment between LFPs and the animal's licking, we bandpass filtered the LFPs around the licking frequency and used the Hilbert transform to extract the amplitude and phase of the peri-lick rhythm (left plots in Figure 3B). The phase of LFPs was plotted using polar histograms (right plot in Figure 3B).

To quantify relationships between licking and LFP phase, we used circular statistics to measure the consistency of the phase angles at the time of licks. We used Rao's spacing test for uniformity, which assesses the directional spread of circular data. We plotted each electrode's location in MFC and shaded the locations by the intensity of the Rao statistic (Figure 4A). Phase entrainment to the lick cycle was concentrated in three longitudinal zones within the prelimbic cortex (0 to 1 mm lateral the midline), the medial agranular cortex ("M2") (1 to 1.5 mm lateral to the midline), and a the border of the medial and lateral agranular cortex (2 to 2.5 mm lateral to the midline). Examples of entrainment at four electrodes (from four different rats) located in each extreme of MFC space (rostral/lateral, rostral/medial, caudal/lateral, and caudal/medial) are shown in Figure 4B.

Remarkably, the LFPs as a population had a mean phase angle near 0 degrees, i.e., at the peak or trough of the neural oscillation (Figure 4C). This distribution was entirely distinct compared to population summaries based on surrogate data (i.e., "licks" derived from shuffled inter-lick intervals). Therefore, the MFC region as a whole showed a relatively similar phase that is suggestive of phase entrainment to the lick cycle.

To examine effects of licking on MFC across spectral frequencies, we used standard time-

frequency analysis measures (EEGlab toolbox: Delorme and Makeig, 2004). Lick-related changes in spectral power across frequencies between 0 and 100 Hz were measured using Event-Related Spectral Power [ERSP] (Figure 5A). Phase consistency at the times of the licks was measured using Inter-Trial Coherence [ITC] (Figure 5B). Surrogate data (shuffled inter-lick intervals) were used to determine if the spectral measures were driven by licking (observed data) or simply elevated during period of licking (shuffled data). This analysis provided evidence for both measures, ERSP and ITC, being elevated at the licking frequency (right panels in Figure 5A,B).

A previous study reported that consummatory-related activity in the MFC is sensitive to satiety (Bouret and Richmond, 2010; see also de Araujo et al., 2006 for related findings on the rodent orbitofrontal cortex). To determine if our data reflected such cross-session effects, we checked for stability in licking-related ERSP and ITC levels over the behavioral sessions. Licks were divided into 10 blocks with either equal numbers of licks or equal amounts of time in the session. Peak levels of ERSP and ITC between 4 and 12 Hz and within one inter-lick interval around each lick were measured over the blocks. No obvious pattern indicating satiation was apparent in this analysis (ITC results are shown in Figure 6). This analysis suggests that entrainment of MFC activity to the lick cycle does not reflect satiety or other cross-session factors.

Rhythmic activity encodes reward value and develops with experience

In a subset of three rats, we recorded neuronal activity as the animals learned to perform the Shifting Values Licking Task (Figure 7). Over the first four days of training, the rats showed

increased licking when the higher value reward was available relative to licking for the lower value option (Figure 7A). Repeated measures ANOVA found a main effect of reward value on licking ($F_{(1,14)}$=32.20, p=0.000057). Tukey's *post hoc* test found evidence for a difference between the number of licks for the high-value versus low-value reward in session 4 (p=0.013), but not session 1 (p=0.935). Also, median inter-lick intervals (ILIs) were reduced from session 1 to session 4 (Wilcoxon rank-sum test from three rats individually: p<0.00001), indicating that rats increased their licking frequency with experience in the task.

Entrainment to licking developed over the training sessions, with clear lick-related oscillatory patterns apparent in the LFPs by the fourth training session (Figure 7B). Event-related potentials were larger for licks that delivered the higher value sucrose reward (blue lines in Figure 7B). The spectral content of the signals was evaluated using the same ERSP and ITC analyses used in Figures 5 and 6. Notably, there was a distinction between high-value and low-value phase-locking to the onset of licking evident in LFP data from session 4, but this signal was not apparent during session 1 (Figure 7C). To capture effects of reward value on the LFPs, we calculated a "value index" for each electrode (Figure 7D). This index was derived from difference between the peak ITC level for the high and low-value licks divided by the peak ITC level for the high-value licks. All electrodes from all rats showed an increase in this index, a result that indicates increased differences in phase-locking for two amounts of reward over the period of training (paired t-test: t(39)=-12.085, p<0.00001).

To further measure changes in the signals associated with the two reward values over sessions, we performed a repeated-measures ANOVA with the peak ITC values as the dependent

variable and the values of the licks and the training sessions as predictors. This analysis found a significant interaction between session and value ($F_{(1,155)}$=22.43, p<0.00001), and Tukey's *post hoc* test found evidence for differences between session 1 versus session 4 high-value lick ITC values (p=0.0016). While these analyses found evidence for significant difference between ITC levels for the high and low-value licks in session 1 (p=0.0093), the difference between ITC levels was much greater in session 4 (p<0.00001).

We also assessed changes in LFP power by performing the same type of repeated-measures ANOVA, using peak ERSP values as the dependent variable. There was a significant interaction between session and reward value ($F_{(1,155)}$=9.991, p=0.0019). Tukey's *post hoc* analyses showed a difference in power from session 1 to session 4 high-value licks (p=0.00035), as well as power for session 4 high and low-value licks (p=0.000043). There was no difference in power between session 1 high-value and low-value licks (p=0.99). These findings are evidence for learning-dependent changes in the encoding of reward value by the event-related power (ERSP) and phase (ITC) to the lick cycle.

MFC spike activity is entrained to the lick cycle and encodes reward value

Simultaneous recordings of spike activity in the three rats tested during the final learning session showed evidence for spike entrainment to the lick cycle as well as an encoding of reward value (Figure 8A). The probability of spiking at the times of licks was below 0.1 for all isolated single-units. Therefore, we re-isolated multi-unit activity [MUA] (N=44) for the analyses reported here and measured the probability of spiking at the times of the higher-value and lower-value

licks. Spikes were more likely to be coicident with the higher-value licks (0.113±0.013, mean±sd) compared to the lower-value licks (0.092±0.009) for 33 of 44 spike recordings (Figure 8B; paired t-test: $t(43)=-3.78$, $p=0.00047$).

Spike-lick coherence was used to further analyze synchronization between spikes and the higher and lower value licks. Results were complicated (and thus not shown graphically), with spikes having major peaks at various frequencies in the beta and gamma ranges, and spike-lick coherence often being significant in those ranges. 33 of 44 units (75%) fired in phase with the higher value licks. 19 units (43%) fired in phase with licks that delivered the lower value fluid. Over all recordings, the level of spike-lick coherence was greater for the higher value licks compared to the lower value licks (paired t-test: $t(43)=4.6$, $p<0.001$).

A much simpler result was obtained by using spike-field coherence to examine synchronization between the spikes and fields (Figure 8C). All 44 MUA recordings exhibited significant levels of spike-field coherence at the licking frequency (lower left plot in Figure 8C). Interestingly, phase was uniformly near $-\Pi$ for these datasets (lower right plot in Figure 8C), indicating that spikes and fields were explicitly out of phase (antiphase). Together, these results suggest that the encoding of reward value as measured in the LFPs was also manifest in lick-entrained spike activity within the rostral MFC.

Reward context, not reinforcement, drives licking-related theta entrainment

The signals described above could reflect the expected reward magnitude (van Durren et al., 2008) and/or the taste properties of the ingested solutions (Jezzini et al., 2013). To examine

these issues, we modified the Shifting Values Licking Task to include a 2-sec period of non-reinforced licking between periods of reward delivery (Figure 9A). This procedure resulted in rats continuously licking at the spout during the non-reinforced blocks of the task (Figure 9B). Six rats were tested in the procedure with neuronal recordings. All rats continued to lick more during these non-reinforced blocks when they could receive the high-value fluid compared to when they could receive the low-value fluid ($t_{(5)}$ = 4.25, p=0.0014 for all high-value context licks against all low-value context licks; $t_{(5)}$=4.87, p=0.0046 for non-reinforced high-value context licks versus non-reinforced low-value context licks; $t_{(5)}$=-4.92, p=0.0044 for reinforced high-value context licks versus reinforced low-value licks).

LFP signals synchronized to reinforced and non-reinforced licks were similar, with the main differences between high-value licks and low-value licks still evident, despite the rats not being rewarded during the non-reinforced blocks. Figures 9C and D show group summaries of the differences in peak ERSP and ITC values at the onset of the reinforced and non-reinforced high-value licks. (We chose to focus on the high-value licks for the analyses due to the increased number of high-value licks emitted during the task, though low-value licks also show the same effect.) There were no major differences in peak ERSP levels for reinforced and non-reinforced licks ($F_{(1,359)}$=2.52, p=0.11), which is also evident in the spectral plots from an example LFP recording shown in Figure 9E.

However, the majority (60 of 91) of the electrodes (from all rats) showed increases in ITC phase-locking values for the non-reinforced high-value licks (Figure 9D). We performed a repeated-measures ANOVA with factors for lick type (reinforced or non-reinforced) and reward

value (high or low) with peak ITC values as the dependent variable. This analysis found evidence for a significant interaction between lick type and reward value ($F_{(1,359)} = 31.94$, $p < 0.00001$). The non-reinforced licks had slightly greater ITC values at the onset of licking (high-value reinforced licks = 0.48, SD = 0.069; high-value non-reinforced licks = 0.51, SD = 0.063), which was also confirmed using Tukey's *post hoc* test (reinforced versus non-reinforced high-value licks, p=0.0002). Spectral plots, shown in Figure 9F, revealed modest increases in phase-locking for the non-reinforced high-value licks, and minimal differences in the phase-locking for the reinforced versus non-reinforced low-value context licks. These findings suggest that the reward expectation (or context), rather than the properties of the delivered fluids, drives reward signaling in the rostral MFC.

Reward signaling depends on the medial frontal cortex

Having established the MFC as signaling reward value through lick-entrained neuronal rhythms, we carried out a reversible inactivation study to determine if the rhythmic activity is generated by neurons in the MFC. (Alternatively, the signals could be generated elsewhere and broadcast to the MFC.) We implanted four rats with a multi-electrode array in one hemisphere and a drug infusion cannula to allow for infusing muscimol in the MFC in the opposite hemisphere (Figure 2A). Cross-hemispheric inactivation was done to allow for recording distant effects of MFC perturbation, and not the effects of a local shutdown of neuronal activity. Two of these rats had precisely aligned electrode arrays and drug cannula (same cytoarchitectural area and layer). Two other rats were not precisely aligned (e.g., cannula in superficial layers and array

in deep layers), and electrophysiological data from those animals were not considered further. In all four rats, we did not observe any major behavioral change in the number of licks emitted after the unilateral muscimol infusions. (This is in contrast to our previous study with bilateral inactivations (Parent et al., 2015a), which clearly alter performance of the task.) The lack of behavioral effects of muscimol allowed us to assess potential electrophysiological changes without overt effects of the inactivations on the animals' licking behavior. There was a marginal decrease in the overall inter-lick intervals under muscimol (Wilcoxon rank-sum test, $p<0.05$), but no other effects of inactivation were apparent (e.g., duration of licking bouts).

In the two rats with aligned electrode arrays and drug cannulas, LFP activity during muscimol inactivations was dramatically altered. Muscimol infusions slightly decreased event-related potentials synchronized to licking (Figure 10A) and dramatically decreased event-related spectral power [ERSP] at the licking frequency (Figure 10B, all electrodes plotted from two rats). This was confirmed in the spectral plots, shown from one example electrode from one rat (Figure 10D). A repeated measures ANOVA on peak ERSP values around the onset of licking found evidence for decreased power at lick-onset between the saline and muscimol sessions ($F_{(1,123)} = 96.09$, $p<0.00001$). Muscimol infusions also decreased the lick-entrained phase-locking in the theta frequency range. As seen in Figure 10C, 28 of 32 electrodes showed decreased phase-locking around the onset of licking. A repeated measures ANOVA found evidence for difference in ITC values for the saline and muscimol sessions ($F_{(1,123)} = 18.17$, $p=0.000039$). Spectral plots from an example electrode (Figure 10E) show diminished phase coherence in the theta frequency range for the high-value licks. The decrease in phase-locking therefore

disrupted the previously evident differential signaling of reward value. Together, this inactivation study established that the reward signals described throughout this study, and also reported for single-value water rewards in Horst and Laubach (2013), are generated by neurons in the rostral MFC.

Discussion

Dynamics of reward signaling in the MFC

We examined how neuronal activity in the rat MFC encodes reward value. The main idea of this study was that rodents lick to consume rewarding fluids and reward values are therefore likely to be encoded in a dynamic manner, in phase with the animal's lick cycle. Initial evidence for this idea was reported by Horst and Laubach (2013), who found MFC neurons that were selectively activated when thirsty rats initiated bouts of licking to consume water. The present study was designed to further examine the dynamics of reward sensitivity of MFC neurons to varying levels of sucrose reward using a MFC-dependent incentive contrast task (Parent et al., 2015a,b). Multi-electrode recordings were made in the rostral MFC as rats consumed relatively higher and lower value rewards that were available in alternating periods of 30 sec (Figure 1). The value of the fluids was encoded by the extent of entrainment of MFC spike activity and field potential activity to the animals' lick cycle (Figures 3-6 and 8). These signals were distributed broadly throughout the rostral part of the MFC (Figure 4). Spectral methods showed that these effects were selective to the animals' licking frequency (right plots in Figure 7C).

In addition to documenting the dynamics of reward coding, we also examined how

reward signals develop with experience, depend on the presence of the rewarding fluids, and depend on processing by MFC neurons. Three experiments were carried out to examine these issues. First, we recorded neuronal activity throughout the period of initial learning and found that entrainment between licking and MFC activity became increasingly apparent with experience in the task (Figure 7). Second, as the rostral MFC is known to have extensive interconnections with the “gustatory” insular cortex (Gabbott et al., 2003), MFC reward coding might simply reflect differences in the tastes of the sucrose solutions. To examine this issue, we modified the task to have periods of non-reinforced licking between each reward delivery. If taste drives MFC signaling, then differences in MFC activity should only occur when the animals were actively ingesting the fluids. Surprisingly, this experiment revealed that the coding of reward value persisted beyond the period of reward delivery (Figure 9), a finding that is suggestive of reward expectation or contextual information. Finally, reversible inactivation methods were used to determine if MFC neurons are necessary for the generation of the reward signals (Figure 10). It is possible that value signals are broadcast to the MFC from other brain areas (e.g. gustatory cortex, amygdala) and MFC neurons themselves have no role in the generation of the lick-entrained reward signals. Perturbations of the MFC, made by infusing muscimol into the MFC in opposite hemisphere, dramatically attenuated MFC signaling in the opposite hemisphere, a finding that directly implicates MFC neurons in the generation of the reward signals.

Together, our findings suggest that the MFC, at least in rodents, accounts for reward information in an action-centric manner (“the value of licking now”) using signals that are

synchronized to ongoing actions. Previous studies of MFC reward signaling have inferred value coding upon temporally sustained activity during the period of reward consumption (e.g. Luk and Wallis, 2009). Our findings suggest that neuronal activity in the rostral MFC is temporally sustained due to the consumption of the rewarding fluids, and does not entirely reflect the abstract properties of rewarding foods and fluids.

Rhythmic activity encodes reward value

Two major rhythms were prevalent in our neuronal recordings. Power was elevated between 2-4 Hz (delta) throughout the performance of the task, but this frequency was not phase locked to the lick cycle (Figure 5) and did not encode differences in reward magnitude (Figure 7). A second major rhythm occurred between 4 and 12 Hz (theta) (Figure 5). The phase of the 4-12 Hz rhythm was consistent from lick to lick (Inter-Trial Coherence) and the strength of this “phase locking” was enhanced when rats consumed the higher value reward (Figure 7). Spike activity was coherent to this frequency range for all multi-unit recordings that were made simultaneously with the field potential recordings (Figure 8). Together, these findings suggest that reward information is encoded by 4-12 Hz rhythmic activity in the rostral MFC.

Theta-range rhythms are a prominent feature of the frontal cortex across species (Cavanagh and Frank, 2014). In rodents, frontal theta signals represent information about behavioral outcomes and performance adjustment (Narayanan et al., 2013; Laubach et al., 2015), interval timing (Parker et al., 2014; Emmons et al., 2016), freezing during fear conditioning (Karalis et al., 2016), and consummatory action (Horst and Laubach, 2013). The

results reported here establish that frontal theta also has a role in reward processing.

Orolingual aspects of MFC function

The prominence of theta in rodent frontal cortex during consummatory and reward-related studies is especially interesting because rats naturally lick at frequencies between 6 and 8 Hz (Weijnen, 1998) and open/close their jaw in the 5-7 Hz range (Sasamoto et al., 1990). These behaviors are controlled by brainstem central pattern generators (CPGs) (Travers et al., 1997). Other orofacial behaviors such as chewing/mastication (Nakamura and Katakura, 1995), breathing, sniffing and whisking (Moore et al., 2013) also occur in a rhythmic manner and are controlled by CPGs in the brainstem (Moore et al., 2014). These subcortical sensorimotor areas receive projections from the rostral MFC (Yoshida et al., 2009; Haque et al., 2010; Iida et al., 2010) and stimulation of rostral MFC has direct effects on orofacial movement (Brecht et al., 2004; Adachi et al., 2008) and breathing (Hassan et al., 2013). In light of these studies, we propose that the rostral MFC serves as “cingulate motor area” (Shima and Tanji, 1998) controlling orolingual actions in a value-based manner.

Reward signals are generated by MFC neurons

Unilateral reversible inactivations decreased theta phase tuning (Figure 10C,E) in the opposite hemisphere, established that these signals depended on neurons within the MFC. These inactivations were unilateral and there were no overt behavioral changes to the animals’ licking behavior during the inactivation sessions. This is in strong contrast to bilateral

inactivations of the same cortical area which temporally fragments licking and eliminates the expression of learned incentive contrast effects in the task (Figures 3 and 7 in Parent et al., 2015a). It is not uncommon for unilateral cross-hemispheric inactivations to show less dramatic effects on behavior (Ambroggi et al., 2008), and it was necessary for our interpretations to have the rats maintain their behavior without normal MFC function. Our findings from the inactivation study bolster evidence for the role of MFC in encoding reward value and suggest that adequate signaling of reward value depends on local activity within the rostral MFC.

Conclusion

We have shown a role for the rostral area of the rat medial frontal cortex in encoding the value of consummatory rewards through theta-range rhythmic activity that is synchronized to the animal's ingestive behavior. These signals could enable control over food-based decisions and self control over eating. These issues have clinical implications for understanding anhedonia in psychiatric diseases such as depression and schizophrenia (Gorwood, 2008) and the loss of control over food intake in obesity (Volkow et al., 2011) and eating disorders such as anorexia (Uher et al., 2004).

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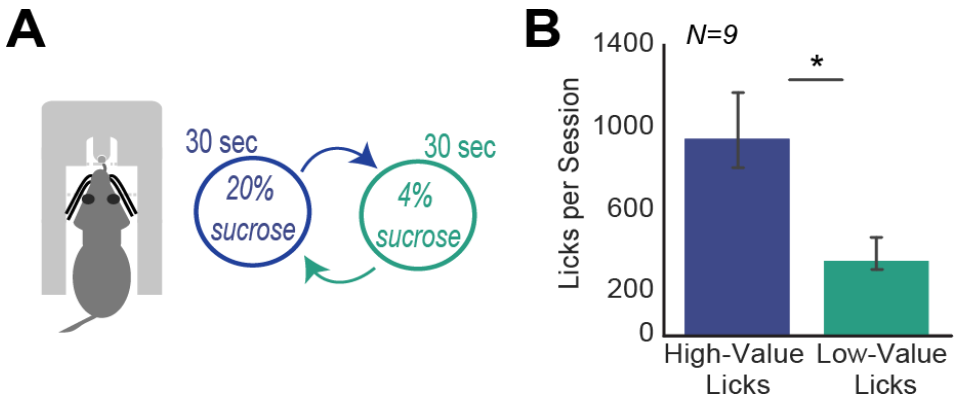

Figure 1: Behavioral task. A. Rats were tested in an incentive contrast procedure called the Shifting Values Licking Task (Parent et al., 2015a). They were required to lick on a spout to receive liquid sucrose rewards. Reward values shift between relatively high (20% wt/vol) and low (4% or 2% wt/vol) concentrations of sucrose every 30 sec. B. Experienced rats (fourth training session) licked more for the high-value sucrose than for the low-value sucrose. Asterisk denotes $p<0.005$ (paired t-test; $t(8) = 4.29$, $p = 0.0026$).

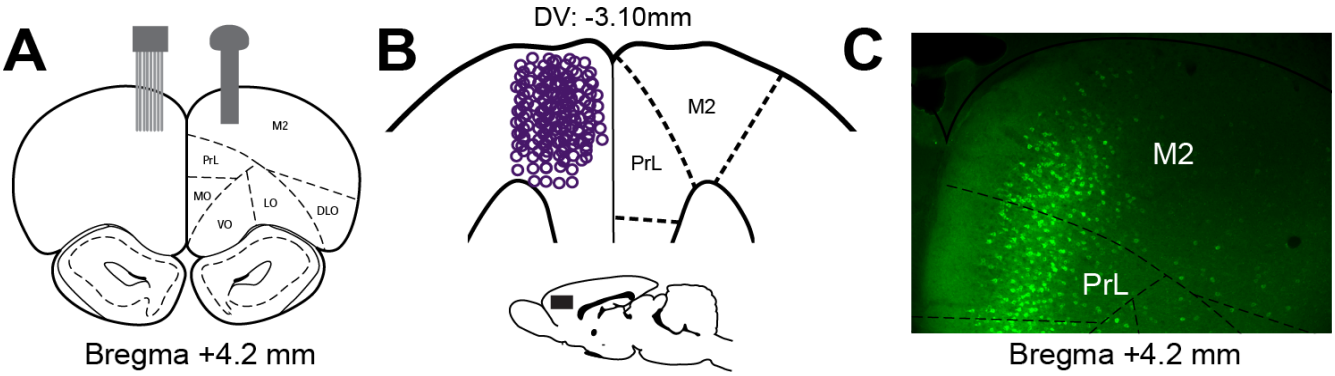

Figure 2: Neuronal recordings. A. All rats (N=11) were implanted with a 16-channel microwire array targeting the rostral medial frontal cortex [MFC] in one hemisphere. A subset of rats (N=4) had a drug cannula implanted in the same cortical area in the opposite hemisphere. B. Locations of recording sites are depicted on a horizontal section from the Paxinos and Watson (1997) atlas. All electrodes were placed within the prelimbic [PrL] and medial agranular [M2] regions. C. Validation of cross-hemispheric connections for this rostral MFC region. Cholera Toxin subunit B with the Alexa Fluor 488 reporter was injected in the rostral MFC of 5 rats. Neurons were labeled in the superficial layers in the opposite hemisphere.

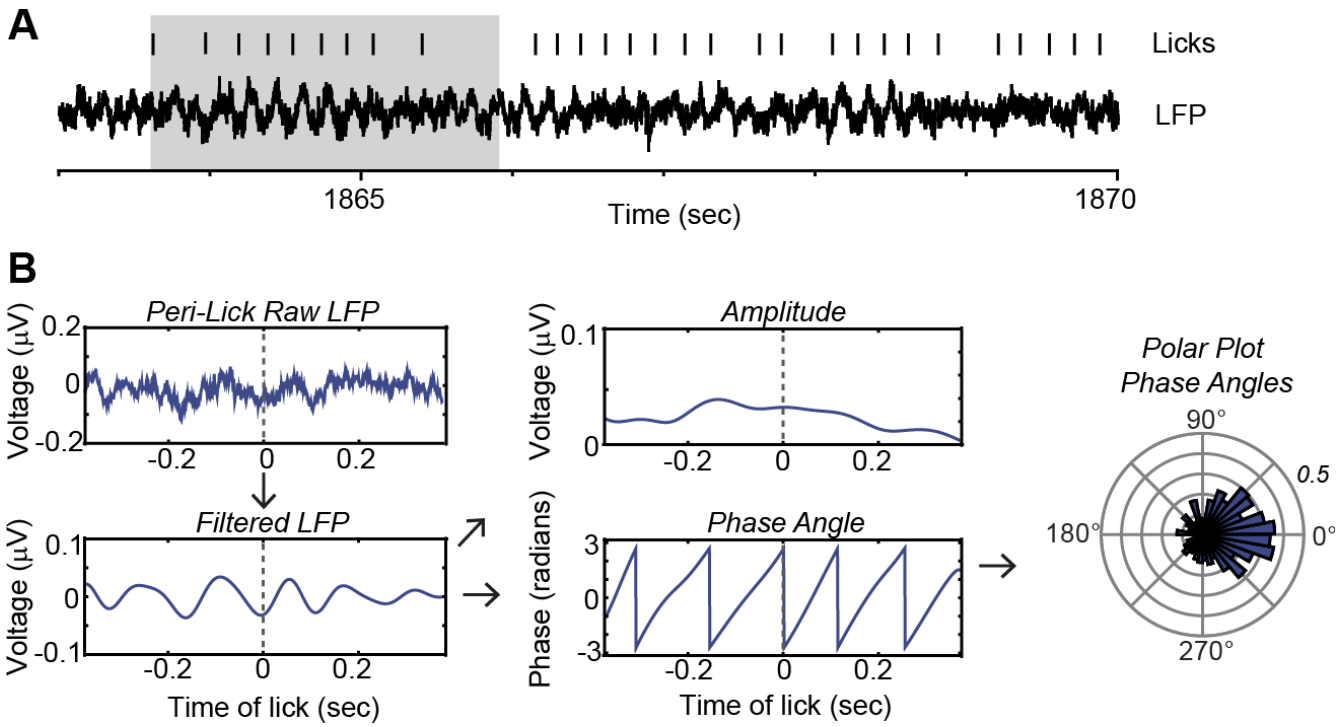

Figure 3: Neuronal activity in the MFC is entrained to the lick cycle. A. An example of a local field potential [LFP] recording shows clear fluctuations at the times of licks (tick marks above the LFP). Gray box denotes delivery of fluid. B. Relationships between LFP signals and licking were assessed by bandpass filtering the LFPs near the licking frequency (defined by the inter-quartile range around the medial inter-lick interval) and applying the Hilbert transform to measure the amplitude and phase of licking-related neuronal activity. Instantaneous phase was plotted using polar coordinates and analyzed with standard methods for circular statistics (Agostinelli and Lund, 2013). See methods for details.

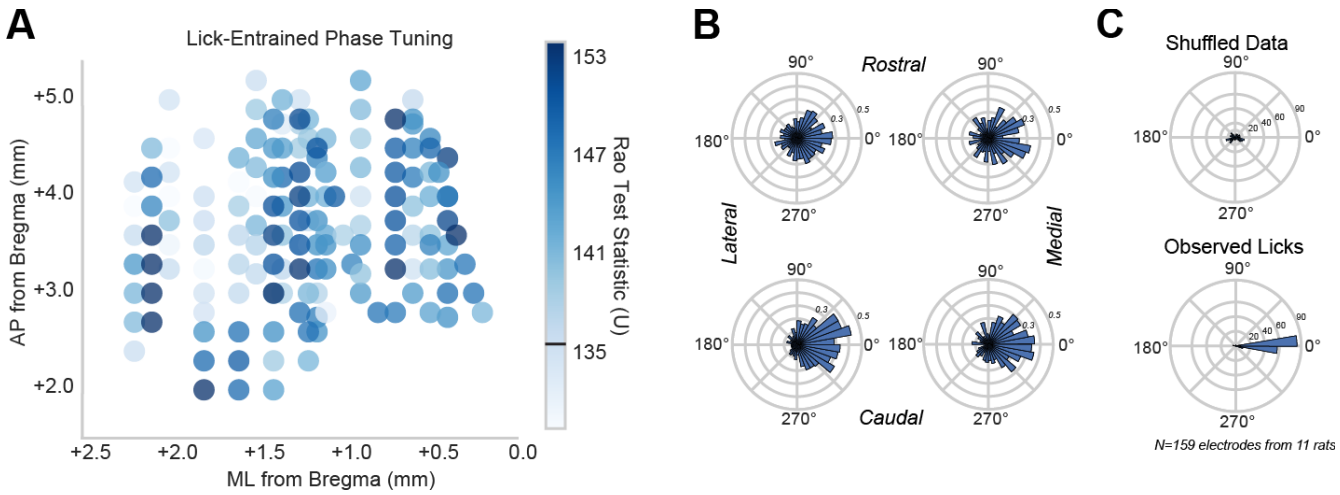

Figure 4: Spatial distribution of entrainment to the lick cycle. A. Spatial plot of phase tuning using the test statistic from Rao's spacing test of uniformity showed no obvious topography of lick-entrainment in the MFC. Individual electrode locations were plotted according to their location in reference to Bregma (N=159 electrodes). Recording sites were depicted as circles colored by the strength of their Rao test statistic [U]. The colorbar shows values of U from the 5th to 95th percentile range over all recording sites. Values above the black bar (near 135) were not uniform ($p<0.05$). B. Polar plots represent phase tuning examples from four spatial extremes of the graph in (A). The most rostral/lateral (top left; $U=134.48$, $p>0.05$), rostral/medial (top right; $U=152.30$, $p<0.001$), caudal/medial (bottom right; $U=153.51$, $p<0.001$), and caudal/lateral (bottom left; $U=147.44$, $p<0.001$) electrodes were chosen. There was no drastic difference among the four locations with regard to phase tuning. C. Group summaries of the mean phase angle at the time of licking from all 11 rats reveal significant phase tuning toward 0 degrees (i.e., peak or trough of the rhythm). These results were compared with phase angles measured from surrogate data (shuffled inter-lick intervals), which did not show evidence for significant phase entrainment.

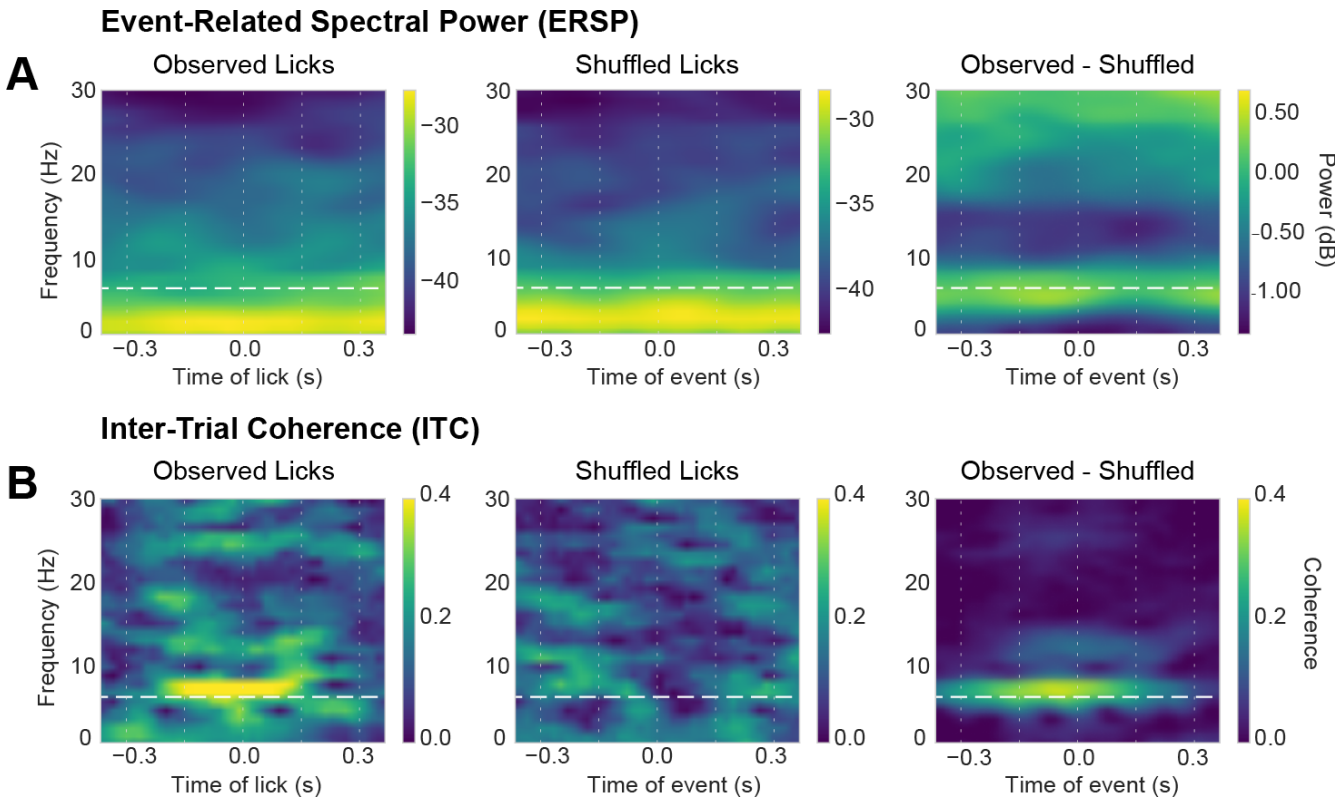

Figure 5: Time-frequency analysis of lick-entrained LFP data. Event-Related Spectral Power [ERSP] (top) and Inter-Trial Coherence [ITC] are shown for a typical LFP recording aligned to the time of licking in the behavioral task. The white horizontal dashed line depicts the median licking frequency. The white vertical dashed lines depict the median inter-lick intervals. ERSP and ITC measures were computed using observed licks (left) and surrogate data (middle), created by shuffling inter-lick intervals. A. Persistent elevated ERSP was notable at very low frequencies (~2 Hz, or delta) for both the observed (upper left) and shuffled (upper middle) events, i.e., was not entrained to the lick cycle. Subtraction of the shuffled ERSP matrix from the observed ERSP matrix revealed elevated power at the licking frequency (horizontal dash line). B. ITC was apparent near the licking frequency over a period of two lick cycles for the observed licks (lower left), but not the shuffled licks ((lower middle). Subtraction of the shuffled ITC matrix from the observed ITC matrix revealed elevated power at the licking frequency (horizontal dash line).

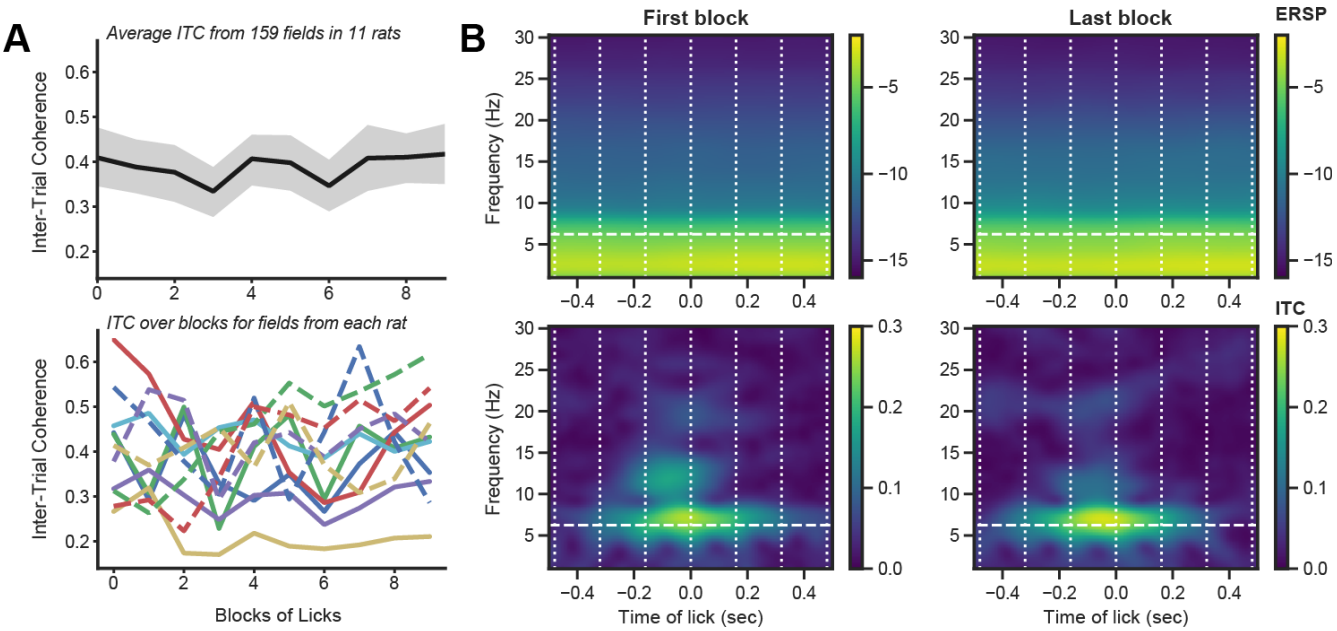

Figure 6: Entrainment was stable over the 30-min test sessions. Sessions were split into 10 blocks with equal numbers of licks and peak event-related spectral power [ERSP] and inter-trial coherence [ITC] were measured in the theta frequency range (4-12 Hz) over the inter-lick interval before and after each lick. A. Group average of peak ITC showed no evidence for a change in this measure over the data sets. Similar results were obtained for ERSP (not shown). B. Traces for peak ITC from each of the 11 rats. C. Grand average of ERSP and ITC for all LFPs in the first and last block. Together, these results suggest that entrainment of MFC LFPs to the lick cycle was not sensitive to cross-session factors such as satiety.

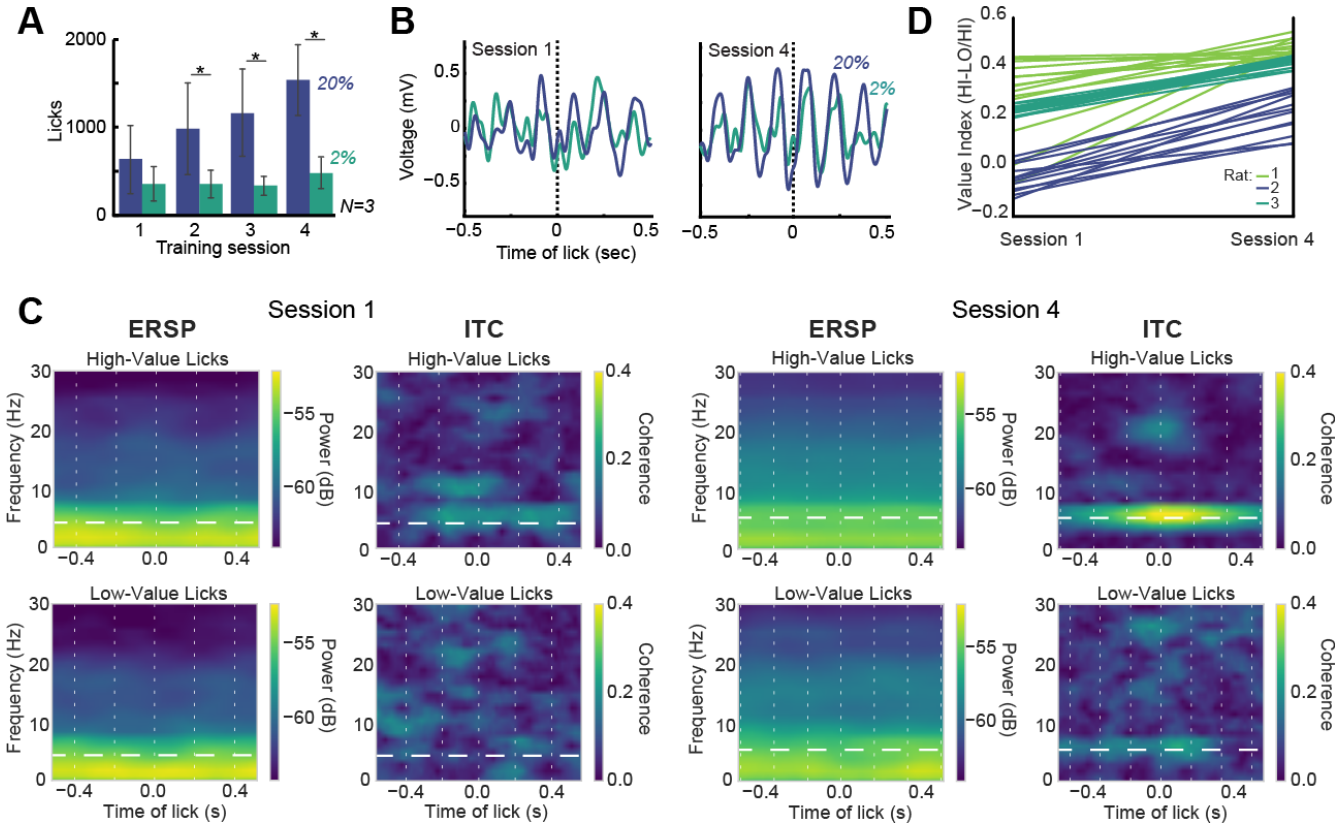

Figure 7: Rhythmic activity develops in MFC with experience and encodes reward value. A. Recordings were made in a subset of three rats as they learned the behavioral task. The rats showed increased licking for the high-value sucrose compared to the low-value sucrose after the first training session and the relative difference in licking increased over the first four training sessions. Asterisk denotes $p<0.05$. B. Neuronal entrainment to the lick cycle developed with experience in the task. For example, event-related potentials increased in size and apparent rhythmicity between the first and fourth training session (blue = higher-value 20% sucrose; green = lower value 2% sucrose). C. Increased entrainment to the lick cycle was also apparent in Inter-Trial Coherence [ITC], which was not apparent in session 1 and specific to licks that delivered high-value sucrose in session 4. (White vertical lines = average inter-lick intervals across the session. White horizontal dashed line = average licking frequency across the session.) D. To capture differences in ITC values for the two types of licks across all recordings, we used a value index, defined as ITC-HI – ITC-LO)/ITC-HI. The index was based on the peak ITC values in a temporal window ranging from one inter-lick interval before lick onset up to 50 ms after the lick and for all frequencies between 4 and 12 Hz ("theta"). As shown in the parallel line plot, in which each line denotes a LFP recording from a distinct electrode, this index was larger in session 4 compared to session 1 (paired t-test: $t(39)=-12.085$, $p<0.00001$).

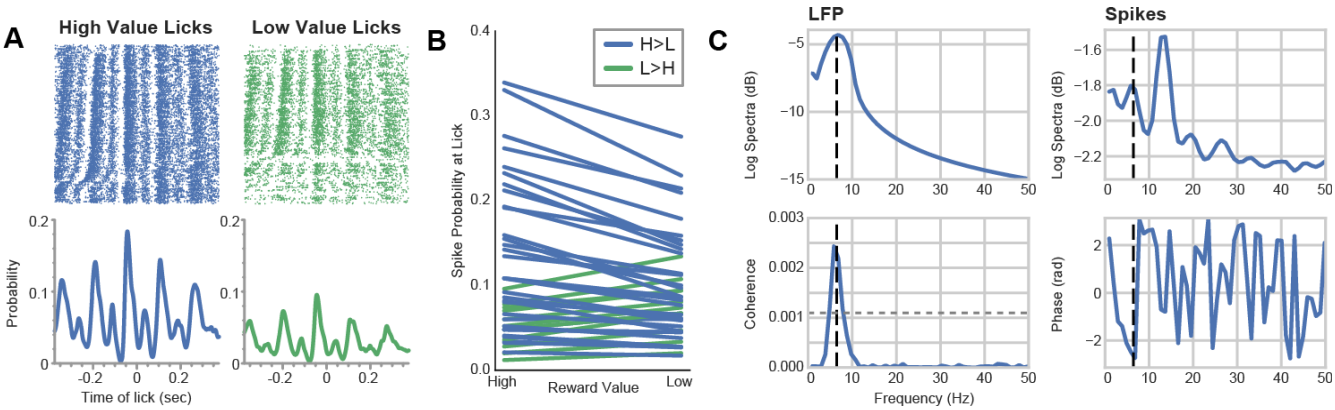

Figure 8: Coherence between spikes and licks reflects reward value. A. Multi-unit spike activity [MUA] was entrained to the lick cycle (high-value licks = blue; low-value licks = green). Rasters were sorted by the latency to the last lick before the lick at time 0, with the shortest preceding intervals at the top of the raster. The high-value licks were sub-sampled for this plot so that neural activity could be compared for the same number of total licks (at time 0). Peri-event histograms (bin: 1 ms, 10-point Gaussian smoothing), below the raster plots, denote the probability of spiking around the times of the licks. B. Group summary for spike probability at times of higher and lower value licks. Blue lines indicate higher spike probability for the higher value sucrose. Green lines indicate higher spike probability for the lower value sucrose. Spike probability was higher at the times of the higher value licks compared to times of the lower value licks (paired t-test: $t(43)=3.78$, $p<0.001$). 33 of the 44 MUA recordings showed higher spike probabilities for the higher value licks. C. Spike-field coherence found that all 44 MUA recordings were entrained to the LFP fluctuations that encoded reward value. Power spectra are shown in the upper row for example LFP and MUA recordings. Peak power was near the licking frequency (black dashed line) for the LFP. The main peak for the spike train was in the low beta range (12-15 Hz) and a second peak was at the licking frequency. Coherence between these signals (lower left plot) was found at the licking frequency (5.96 Hz), at a level approximately twice the 95% confidence interval. The phase between the spikes and fields at the licking frequency (lower right plot) was near $-\Pi$, suggesting that the spikes and fields had an antiphase relationship.

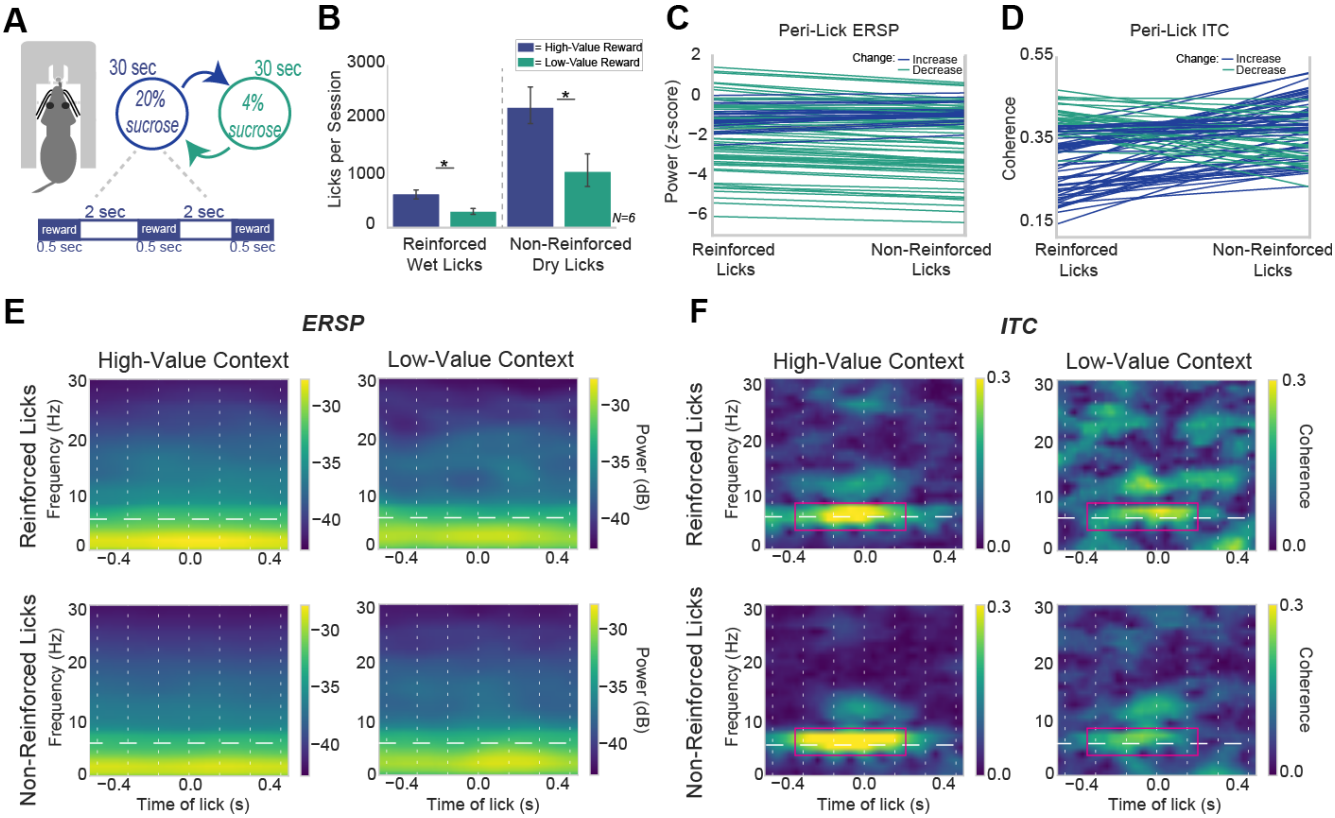

Figure 9: Reward context, not reinforcement per se, drives reward signaling. A. The Shifting Values Licking Task was modified to include a 2 sec period between reward deliveries. This period allowed for non-reinforced licks (dry licks at the spout) to be recorded within the 30 sec states of high or low-value sucrose availability. B. Group summary of total licks (N=6) showed that rats licked during the non-reinforced blocks and licked more in the higher-value blocks. Asterisk denotes $p<0.05$. C. Peak ERSP values for reinforced versus non-reinforced licks during the high-value blocks. Lines are colored by their direction (increase or decrease in power). There was no difference in power for reinforced versus non-reinforced licks ($F_{(1,359)}=2.52$, $p=0.11$). D. Peak ITC values for reinforced versus non-reinforced licks during the high-value blocks. The majority of LFPs showed increased phase-locking to non-reinforced licks (blue lines), while electrodes from two rats show a slight decrease in phase-locking for non-reinforced licks (green lines). Overall group summaries show an increase in phase-locking for the non-reinforced licks ($F_{(1,359)} = 31.94$, $p<0.00001$). E,F. Example of time-frequency analysis of a LFP from a rat that showed decreased ERSP and ITC (magenta box) when the rat licked in the lower-value context. ITC was higher near the licking frequency when the higher value reward was available, regardless if the licks were reinforced or not. Horizontal white lines indicate the within-session licking frequencies and vertical white lines indicate the inter-lick intervals for each session.

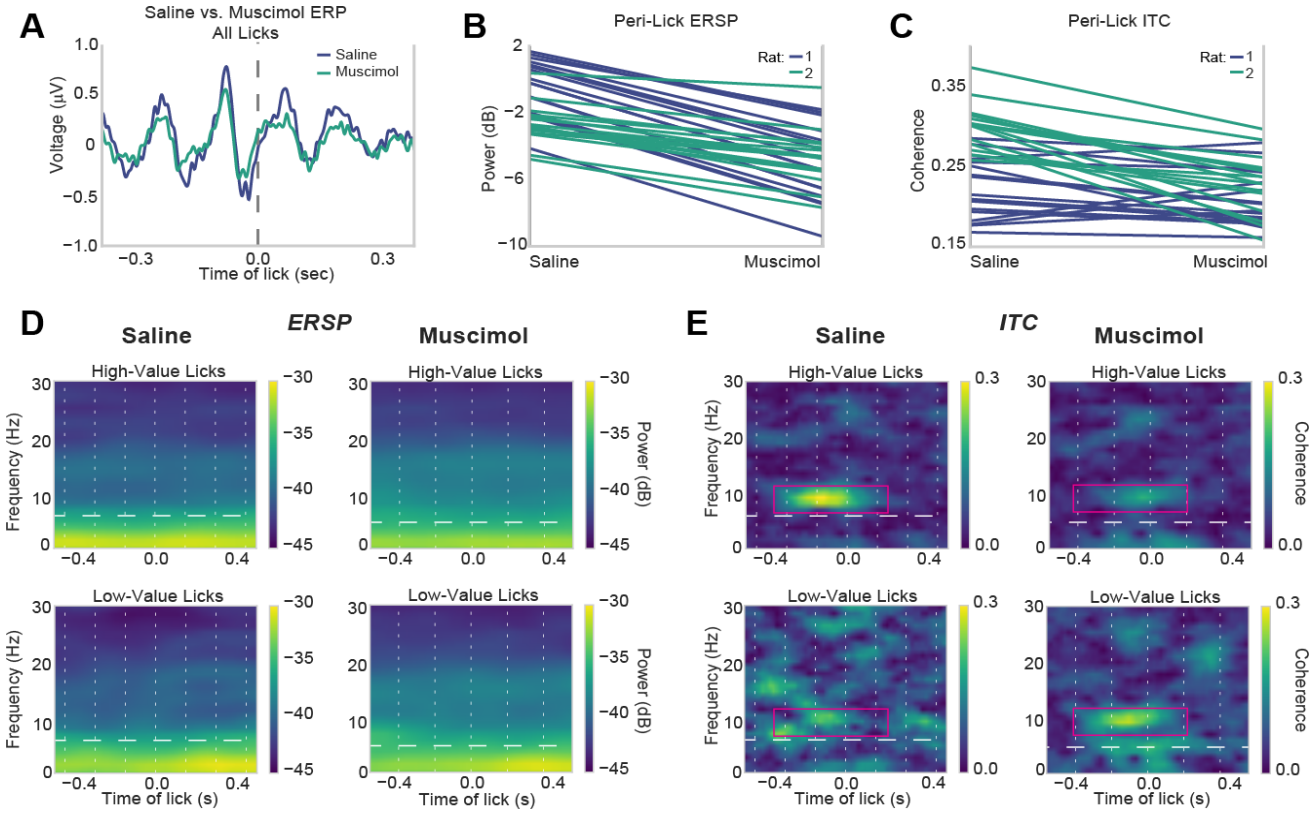

Figure 10: Reward signaling depends on neuronal activity in the MFC. Rats were tested with an electrode array in one hemisphere and an infusion cannula in the other, which was used to infuse either PBS or muscimol. A. Event-Related Potentials [ERP] from the saline (blue line) and muscimol (yellow line) sessions showed a similar overall time course around the licks. B. All electrodes showed a decrease in peak event-related spectral power [ERSP] at the licking frequency for higher value licks during the muscimol sessions compared to the PBS sessions ($F_{(1,123)} = 96.09$, $p<10^{-5}$). C. Likewise, there was a reduction in peak inter-trial coherence [ITC] at the licking frequency for 28 of 32 electrodes ($F_{(1,123)} = 18.17$, $p=3x10^{-5}$). D,E. Example of time-frequency analysis. Effects were specific to licks for the high-value reward. Horizontal white lines indicate the within-session licking frequencies and vertical white lines indicate the inter-lick intervals for each session.