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**The rat medial frontal cortex controls pace, but not breakpoint,
in a progressive ratio licking task**

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Abstract

The medial frontal cortex (MFC) is crucial for selecting actions and evaluating their outcomes. Outcome monitoring may be triggered by rostral parts of the MFC, which contain neurons that are modulated by reward consumption and are necessary for the expression of relative reward value. Here, we examined if the MFC further has a role in the control of instrumental licking. We used a progressive ratio licking task in which rats had to make increasing numbers of licks to receive liquid sucrose rewards. We determined what measures of progressive ratio performance are sensitive to value by testing rats with rewards containing 0-16% sucrose. We found some measures (breakpoint, number of licking bouts) were sensitive to sucrose concentration and others (response rate, duration of licking bouts) were not. Then, we examined the effects of reversibly inactivating rostral (medial orbital) and caudal (prelimbic) parts of the MFC. We were surprised to find that inactivation had no effects on measures associated with value (e.g. breakpoint). Instead, inactivation altered behavioral measures associated with the pace of task performance (response rate and time to break). These effects depended on where inactivations were made. Response rates increased and time to break decreased when the caudal prelimbic area was inactivated. By contrast, response rates decreased and the time to break increased when the rostral medial orbital cortex was inactivated. Our findings suggest that the medial frontal cortex has a role in maintaining task engagement, but not in the motivational control of action, in the progressive ratio licking task.

Key words: sucrose, reward, licking, muscimol, prefrontal, orbitofrontal, rat

Neural recording studies, in rodents and primates, have established that the medial frontal cortex (MFC) is crucial for action selection and initiation and evaluating the outcome of an action (Narayanan & Laubach, 2006; Simon et al., 2015; Sul et al., 2010). For example, MFC neurons signal when errors occur in a simple reaction-time task and the firing rates of these neurons predict changes in action initiation on post-error trials (Narayanan et al., 2013). Computational models of these signals (Bekolay et al., 2014) suggests that reward feedback is crucial for terminating monitoring, and a number of studies have demonstrated the existence of feedback signals during the periods of reward consumption (Horst & Laubach, 2012, 2013; Kargo et al., 2007; Miyazaki et al., 2004; Petykó et al., 2009; Pratt & Mizumori, 2001; Takenouchi et al., 1999).

It turns out that these signals are directly coupled to rats' licking behavior. Horst & Laubach (2013) characterized consumption-related activity using precise measurements of the times when thirsty rats emitted licks to consume water rewards. Fluid was delivered under a variable-interval schedule in this experiment, and rats were not required to do anything other than lick to receive fluid. Many neurons changed their firing rate when rats initiated sustained bouts of licks. The changes in firing rate were accompanied by strong phase locking of simultaneously recorded field potentials to the onset of licking bouts. These signals were especially concentrated in the rostral half of the MFC, in the medial orbital cortex. This area is also known as the medial orbitofrontal cortex or "mOFC" (Fuchs et al., 2004; Izquierdo, 2017).

To determine if this region of the MFC is crucial for value-guided consummatory actions, Parent et al. (2015a) used a behavioral procedure based on classic studies of incentive contrast using liquid sucrose by Flaherty (1999). In 30 minute test sessions, hungry rats had access to relatively high (20%) or low (4%) concentrations of sucrose over alternating periods of

30 sec. The rats learned over days to maintain licking when the high concentration of sucrose was available and to suppress licking when the low concentration was available. The procedure revealed incentive contrast effects, e.g. rats lick more for the high concentration when it is presented in alternation with the low concentration compared to sessions with only the high concentration of sucrose. Reversible inactivation of the medial orbital area, but not the prelimbic area, reduced licking selectively for the high concentration of sucrose and eliminated the incentive contrast effect (Parent et al., 2015a).

To determine if the rostral part of the MFC encodes information about the value of available rewards, Amarante et al. (2017) recorded neural activity in MFC as rats performed in the incentive contrast procedure. They found that the strength of phase locking of neural activity to the lick cycle was proportional to the concentration of sucrose that was consumed. This was the first evidence that neurons in the medial orbital region track reward value on a lick-by-lick basis. Together, the studies by Parent et al. (2015a) and Amarante et al. (2017) suggest that the rostral MFC (medial orbital cortex) mediates a selective drive over the consumption of highly valued fluids and foods.

The present study examined how these findings translate to the control of instrumental licking using the progressive ratio licking procedure of Sclafani & Ackroff (2003). This procedure requires rats to emit an increasing number of instrumental (dry) licks prior to receiving liquid sucrose. Dry licking prior to fluid receipt was found to elicit strong phase locking between licks and MFC field potentials in one of the experiments reported by Amarante et al. (2017). Moreover, the strength of phase locking encoded the value of the forthcoming sucrose reward. Based on this finding, we expected to find that reversible inactivation of the MFC disrupts value-guided control in the progressive ratio licking procedure, e.g. reducing the

breakpoint. Furthermore, based on the Parent et al. (2015a) study, we expected to find that disruptions of instrumental licking occur following inactivation of the medial orbital cortex, and not the prelimbic cortex.

Two experiments are reported below. First, we examined how the concentration of sucrose provided for completing response ratios in the progressive ratio licking task affected a number of “instrumental” and “consummatory” measures. The instrumental measures were the breakpoint, the average response rate (Bizo & Killeen, 1997), the time taken to reach the breakpoint, and the percentage of time engaged in responding. The consummatory measures were the frequency and variability of licking, the number and duration of licking bouts. We analyzed the effects of sucrose concentration on these variables on their own (univariate analyses) and with regard to each other (multivariate analysis), as in Killeen & Hall (2001). This study was necessary because the original study by Sclafani and Ackroff (2003) only examined effects of sucrose concentration on breakpoint. Second, after identifying which behavioral measures were sensitive to sucrose concentration, we reversibly inactivated locations in the medial orbital and prelimbic cortices and evaluated how perturbations of these regions altered progressive ratio responding. If the medial orbital, but not the prelimbic, cortex selectively mediates value-guided control over progressive ratio licking, then reversible inactivation of the medial orbital cortex should decrease behavioral measures that co-vary with reward value (and the associated cost of acquiring the fluid), such as breakpoint, and inactivation of the prelimbic cortex should have no effects on progressive ratio licking. Our prediction of reduced breakpoint following inactivation of the medial orbital cortex is supported by one recent study (Gardner et al., 2018), but not some others (Kheramin et al., 2005; Gourley et al., 2010), that used traditional progressive ratio designs.

Methods

Procedures were approved by the Animal Use and Care Committee at American University and conformed to the standards of the National Institutes of Health.

Subjects

Experiment 1: Effects of Sucrose Concentration. Twelve female Sprague-Dawley rats (250-300g) were purchased from Envigo, used as control animals from a brief home cage dietary study in which the animals consumed standard chow, and were then re-appropriated for use in the present study. An additional 10 male Long-Evans rats (400-500g) were purchased from Charles River and used exclusively for the present study.

Experiment 2: Effects of Reversible Inactivation of the MFC. A total of 35 male rats (Charles River) and 5 female rats from Experiment 1 were used in the study. Twenty-five rats were implanted with cannulae targeting the prelimbic cortex. Twenty of these rats survived surgery and the subsequent period of recovery (one week). Fifteen rats were implanted with cannulae targeting the medial orbital cortex. Eight of these rats survived surgery and the period of recovery. Eight rats were removed from the study due to inability to confirm cannula placement or infusion of drug. The male rats weighed 400-480g and the female rats weighed 250-300g at the time of testing. Twenty rats in total received muscimol infusions.

For both experiments, animals were housed individually and kept on a 12/12 h light/dark cycle. Animals had regulated access to food to maintain their body weights at approximately 90% of their free-access weights. Rats typically received 14–18 g of food each day at 4 pm and were weighed daily throughout the period of testing in the progressive ratio licking task. Animals had free access to water up to one hour before the session start and immediately upon

returning to the home cage. Regulated access to food, but not water, has been shown to be crucial for establishing the reinforcement value of liquid sucrose (Reilly, 1999).

Apparatus

All animals were trained in sound-attenuating behavioral boxes (ENV-018MD-EMS: Med Associates) containing a single horizontally placed spout (5/16" sipper tube: Ancare) located on one wall at 6.5 cm from the floor and a white LED light 4 cm above the spout. The behavioral devices were controlled and data were collected using custom-written code for the MedPC system, version IV (Med Associates). Licking was tracked using a photobeam lickometer (ENV-251M: Med Associates). Solution lines were connected to 60 ml syringes and solution was delivered via single speed pumps (PHM-100; Med Associates) which drove syringe plungers. The pumps delivered 0.180 ml per activation over 2 seconds.

Behavioral Procedure

Rats were first given access to 25 ml of 16% sucrose in their home cage once a day over a two day period. They were then acclimated to the operant chamber with unrestricted access to liquid sucrose from the lickometer in 30 minutes behavioral sessions. Fluid presentation was indicated by activation of the fluid pump and illumination of a 5 mm white or green LED located above the lickometer. Once the rats licked more than 1000 times in each of two consecutive behavioral sessions, the rats were trained on the progressive ratio licking task as described in Sclafani & Ackroff (2003). Briefly, the rats were tested with a PR-1 schedule in which the number of instrumental licks required to obtain each successive reinforcement started at 20 and increased by 1 (i.e., 20, 21, 22, etc.). Each reward delivery was paired with the

illumination of the LED above the lickometer. The session was terminated if the rat stopped licking for 3 minutes. Rats were trained until they reached stable breakpoints, determined over a 3-day sliding window (i.e. standard deviation of 10 trials or fewer and the breakpoints were not increasing over that window). For experiment 1, rats experienced sessions with various concentrations of sucrose (0%, 2%, 4%, 8%) provided at the end of each response ratio. Each level of sucrose was tested in a separate test session. Test sessions were interleaved over days with presentations of 16% sucrose. For experiment 2, all sessions used 16% sucrose.

Surgery

Anesthesia was induced by an injection of diazepam (5 mg/kg, IP) and maintained with isoflurane (4.0%; flow rate 4.5 cc/min). Standard stereotaxic methods were used to implant 26-gauge guide cannulae (PlasticsOne) bilaterally along the rostral-caudal axis of the medial frontal cortex. Implants targeted the prelimbic (3.2-3.7mm AP) and medial orbital (4.2-4.7mm AP) cortices. Depth was adjusted according to AP position and ML position was calculated using a posterior angle of 12° and a lateral angle of 30° ($\pm$ 1.0 to 1.4mm). Infusions were made using 33-gauge cannulae (PlasticsOne), which extended 0.5 mm past the end of the guide cannulae.

Behavioral Procedures in Reversible Inactivation Studies

Rats were allowed 14 days of recovery before being tested, after which they were run daily until they reached stable breakpoints that were similar to their pre-surgery breakpoints. Drug infusions were done under isoflurane anesthesia as in previous studies from our lab (e.g. Parent et al., 2015a). To acclimate the rats to testing after gas anesthesia, they were anesthetized for 10 minutes one hour prior to testing for 1-2 sessions. On test days, rats were infused with 0.5

166 μL of either physiological saline or 1 $\mu\text{g}/\mu\text{L}$ muscimol (Tocris). Infusions were done with a flow
167 rate of 0.35 $\mu\text{L}/\text{min}$. Infusion cannulae were left in place for 2 minutes after the infusions to
168 allow for diffusion. Animals were tested 60 minutes after infusion.

170 **Confirmation of Cannula Placement**

171 At the end of all experiments, animals were anesthetized with isoflurane gas and injected
172 intraperitoneally with Euthasol (100 mg/kg). Animals were transcardially perfused with 200 ml
173 of chilled (4°C) physiological saline solution followed by 200 ml of chilled (4°C) 4%
174 paraformaldehyde. Brains were removed and cryoprotected using a solution containing 20%
175 sucrose, and 20% glycerol. Brains were then cut into 50 μm -thick coronal slices using a freezing
176 microtome (Hacker). Brain sections were mounted onto gelatin-coated slides and Nissl stained
177 via treatment with 0.05% thionin. Thionin-treated slices were dehydrated through a series of
178 alcohol steps, then covered with Clearium and coverslipped. Sections were imaged using a
179 Tritech Research scope (BX-51-F), Moticam Pro 282B camera, and Motic Images Plus 2.0
180 software. The most ventral point of the injection bolus was compared against (Paxinos &
181 Watson, 2007) to estimate brain atlas coordinates.

183 **Data Analysis**

184 Behavioral data were saved in standard MedPC data files and were analyzed using
185 custom-written code in the Python and R languages.

186 *Breakpoint* was defined as the final completed response ratio before the rat stopped
187 licking for at least 3 min. *Time to break* was defined as the total time performing the task (in
188 minutes). *Response rate* was defined as the number of required responses divided by the time (in

minutes) to complete the ratio (Bizo & Killeen, 1997). This measure did not include the period of fluid delivery (0.5 sec) upon completion of the ratio and, unlike studies using food pellets, rats do not stop licking after receiving fluid in the progressive ratio licking task (Sclafani & Ackroff, 2003) (There is no “post-reinforcement pause”).

Microstructural measures of licking behavior were analyzed for all licks with inter-lick intervals longer than 0.09 sec (briefer intervals can occur rarely due to the tongue breaking the photobeam twice on one lick cycle). Analysis of inter-lick intervals excluded intervals longer than 1 sec, which occur when rats pause in licking to swallow, breathe or groom. Detection and quantification of licking bouts were done as in previous studies (Parent et al., 2015a).

Specifically, *bouts* were defined as having at least three licks within 300 ms and with an inter-bout interval of 0.5 s or longer. We measured the median *duration of bouts* and the *number of bouts* for each testing session. Given differences in bout durations and inter-bout intervals across rats, we calculated the *percent of time in bouts* as the sum of all bout durations divided by the total experimental time (first to last lick), multiplied by 100.

Due to the long-tailed nature of distribution of inter-lick intervals, a robust measure of the central tendency (or mean) was estimated using the M-estimator method (Huber & Ronchetti, 2009). This metric also estimates the spread of the distribution, equivalent to the standard deviation. These estimates were used to define a robust measure of the coefficient of variation, i.e. the standard deviation divided by the mean. The *statsmodels* library for Python was used for these estimates.

The collection of *instrumental* (breakpoint, total licks, time to break, and response rate) and *consummatory* (M-estimator and robust spread of inter-lick intervals, the median duration of bouts, the number of bouts, and the percent of time in bouts) measures were analyzed for each

testing session in Experiments 1 and 2. The sensitivities of these measures to sucrose concentration (Experiment 1) and the inactivation of the medial frontal cortex (Experiment 2) were evaluated using multivariate and univariate analysis of variance (ANOVA) and linear regression. Data from inactivation sessions were normalized relative to control sessions to reduce effects of inter-subject variability. Figures depict a %-change in behavioral measures: for example, for variable X, $(X_{\text{Muscimol}} - X_{\text{Saline}}) / X_{\text{Saline}}$.

Exploratory data analysis indicated unequal variance over levels of sucrose concentration and between saline and muscimol sessions for several behavioral measures. This was confirmed using the Ansari-Bradley two-sample test for a difference in scale parameters (available in the *scipy* library for Python). As a result, all statistical testing used permutation methods (2000 permutations per test).

Multivariate ANOVA was conducted using the R function *manova*, based on Wilks' lambda. Empirical p values were calculated for the approximate F statistic using permutations of the outcome variables (sucrose concentration or drug state of cortex). Univariate ANOVA was done using the *permuco* library for R, which implements methods from (Kherad-Pajouh & Renaud, 2015). Posthoc testing used conditional inference tests from the *coin* library for R, using a wrapper from the *rcompanion* library. p-values were adjusted for false discovery rates.

Methods for robust linear regression (Theil-Sen estimator from the *scipy* library for Python) were used to evaluate the slope of fitted lines over the levels of sucrose (Experiment 1). Sucrose concentrations were transformed using a log2 with water reset to a value of 0 (this was necessary because the log2 of 0 is negative infinity). In the Results section, we only reported linear dependencies for the behavioral measures (over levels of sucrose concentration) if the estimated 95% confidence interval for the slope was offset from 0.

Correlations among the behavioral measures were evaluated using the *corrplot* library for R, and clusters of functional related measures were estimated using hierarchical cluster analysis (*hclust* function in R). Relations among variables were further evaluated using principal components analysis with varimax rotation, using routines in the *psych* library for R including methods for estimating the dimensionality of the data set (*VSS* function, Revelle & Rocklin, 1979).

Results

Experiment 1: Effects of Sucrose Concentration

The effects of sucrose concentration on nine instrumental and consummatory measures was evaluated in Experiment 1. Twenty-two rats (12 females and 10 males) were tested. A multivariate ANOVA with permutation testing was significant for the effect of sucrose concentration on these measures ($F(4,105)=5.131$; $p=0.0000$). There were major differences in body mass in the males ($428.9625 \pm 40.3203\text{g}$, Bayesian estimate of mean $\pm$ 95% conf. int.) and females ($253.0833 \pm 14.7540\text{g}$) (Welch's t-test, $t(1,20)=19.7734$, $p=1.3422\text{e-}14$). Exploratory data analysis suggested that interactions between sex/mass and sucrose concentration were apparent for some behavioral measures (e.g. breakpoint, total licks, number of bouts) only at the highest concentration of sucrose (16%). This impression of the data was supported by multivariate ANOVA (interaction between sex/mass and all sucrose concentrations: $F(4,100) = 1.5977$, $p=0.01883$; interaction without 16% sucrose: $F(3,80) = 1.1122$, $p=0.3277$). Results are reported below without regard to sex/mass.

Exploratory data analysis further suggested lower variance for most measures when rats were tested with water compared to any amount of sucrose (Figure 1C-D and 2D-F). These

effects were confirmed using a non-parametric test for homogeneity of variance (Ansari-Bradley test with Bonferroni correction, i.e. $p < 0.0055$), which was significant for all variables except the response rate, the number of bouts, and the inter-lick interval measures (mean and coefficient of variation). For this reason, permutation testing was used in the analyses reported below.

Univariate Analyses of Behavioral Measures

Three of the instrumental measures increased in a linear manner with sucrose concentration: total licks (Figure 1C), breakpoint (Figure 1D), and time to break (Figure 1E). The 95% confidence intervals of the slopes of the robustly fitted regression lines were offset from 0 (slopes in behavioral units: total licks – 866.75 licks; breakpoint – 13.0 breakpoint ratios; time to break – 4 minutes). ANOVAs found evidence for significant variation of total licks ($F(4,105)=22.31$, $p=0.0005$), breakpoint ($F(4,105)=25.09$, $p=0.0005$), and time to break ($F(4,105)=12.49$, $p=0.0005$) over the levels of sucrose. Posthoc tests showed that rats licked more, had higher breakpoints, and worked longer in the task when they received any level of sucrose compared to water ($p < 0.05$ with correction for false discovery rates). Furthermore, posthoc tests showed that rats licked more and achieved higher breakpoints when working for 16% sucrose compared to 2% and 4% sucrose. Response rate (Figure 1F) did not vary with sucrose concentration ($F(4,105)=3.017$, $p=0.0235$, greater than the Bonferroni corrected p-value of 0.0055).

Two of the consummatory measures increased in a linear manner with sucrose concentration: the number of bouts emitted (Figure 2E) and the percent of time engaged in licking (Figure 2F). ANOVA found evidence for significant variation of the number of bouts ($F(4,105)=14.52$, $p=0.0005$) and the percent of time engaged in licking ($F(4,105)=36.65$,

p=0.0005) over the levels of sucrose. Posthoc tests showed that rats emitted more bouts and spent more time engaged in licking when they received any level of sucrose compared to water (p<0.05 with correction for false discovery rates). Posthoc tests further showed that these measures were also sensitive to differences between sessions with 16% sucrose compared to 2% and 4% sucrose and sessions with 8% and 2% sucrose. The 95% confidence intervals for the slopes of robustly fitted regression lines for these variables (not shown) were offset from 0 (slopes in behavioral units: number of bouts – 26.33333 bouts; percent of time engaged in licking – 4.77168 percent).

Effects of sucrose concentration on the other consummatory measures were more subtle. ANOVA suggested that the variation of inter-lick intervals (Figure 2C) was sensitive to sucrose concentration ($F(4,105)=6.434$, $p=0.0005$). Posthoc tests found that this measure was higher when rats worked for water compared to all levels of sucrose. ANOVA did not find evidence for the other consummatory measures (duration of licking bouts – $F(4,105)=2.893$, $p=0.0235$; Average inter-lick interval – $F(4,105)=2.942$, $p=0.022$) being sensitive to sucrose concentration. The empirical p values for these two measures were greater than the Bonferroni corrected p-value (0.0055) and no posthoc comparisons were significant.

Correlation Matrix and Hierarchical Cluster Analyses of Behavioral Measures

The analysis reported above divided the behavioral measures into distinct categories associated with instrumental vs consummatory aspects of performance. It is possible that there were correlations between the measures that did not fall neatly into these categories. To evaluate this possibility, we examined the correlation matrix for the set of nine behavioral variables

(Figure 3A). The matrix strongly suggested that there were two or three clusters of correlated variables, and these did not segregate into groups defined as instrumental and consummatory.

We then used hierarchical cluster analysis (Ward's method) to partition the data set. Several common metrics for evaluating the dimensionality of the data were applied (scree plot, number of eigenvalues > 1 , Very Simple Structure criterion – Revelle & Rocklin, 1979), and suggested there were three significant dimensions (clusters) in the data set. The results of a three-way clustering of the correlation matrix are shown in Figure 3A.

The first cluster was comprised of the average inter-lick intervals and variability of the intervals (first two columns and rows). There were large positive correlations between these measures (Pearson correlation, 0.97156) and smaller negative correlations between each of these measures and the other behavioral variables. The second grouping included some of the instrumental measures (breakpoint, total licks, and time to break) and two consummatory measures (percent of time in bouts and number of bouts). These measures showed strong pairwise correlations with each other (>0.8) and much weaker correlations with the other measures (e.g., correlation between breakpoint and response rate was 0.21738). The third grouping was comprised of the instrumental measure response rate and the consummatory measure bout duration (correlation of 0.52657). These variables also showed positive negative correlations with the percent of time spent licking (0.54660 for response rate and 0.43217 for bout duration), but not the other instrumental measures (e.g. the correlation between for bout duration and breakpoint was 0.26654).

To relate the correlations back to univariate ANOVAs described above, we plotted the F statistics for each behavioral measure below its column in the correlation matrix in Figure 3B. The leading predictor for the effects of sucrose concentration was the percent of time spent

licking. Breakpoint and total licks were the next two leading predictors of sucrose concentration. All three of these measures fell into a common cluster, suggesting that there was a collection of correlated behavioral measures that track reward value (or the cost in acquiring fluid) in the progressive ratio licking task.

Principal Component Analyses of Behavioral Measures

Cluster analysis is designed to find groups in multivariate data sets, but is not easily incorporated into an analysis such as ANOVA. For this reason, we used principal component analysis (PCA), with varimax rotation, to evaluate relationships between sucrose concentration and functional clusterings of the behavioral measures. A scree plot of the eigenvalues (i.e. how much covariance is account for by each PC) is shown in Figure 3C. Only the two leading components (PCs) accounted for more variance than a single behavioral measure (dashed line at eigenvalue of 1).

The same functional clustering found by hierarchical cluster analysis was found by PCA (Figure 3D). The leading component, PC1, was associated with three “instrumental” measures (breakpoint, time to break, and total licks) and two “consummatory” measures (number of bouts and percent of time in bouts). The second component, PC2, was associated mostly strongly with response rate, duration of bouts, and the percent of time in bouts. Other measures were weakly correlated in a positive (e.g. breakpoint) or negative (time to break) with these measures. The third component, PC3, was strongly associated with the two measures of licking frequency and negatively associated with most other measures. By normalizing the eigenvectors (lower plot in Figure 3D), it became clear that only PC1 accounted for major variance across the set of behavioral measures.

We computed scores for the two major PCs and evaluated them using ANOVA. Both components were sensitive to differences in sucrose concentration (Figure 3 E-F; PC1 – $F(4,105)=19.42$, $p=0.0005$; PC2 – $F(4,105)=3.191$, $p=0.016$). Posthoc comparisons suggest that scores for PC1, similar to some of the univariate measures described above, denoted differences in sessions with water compared to sessions with any level of sucrose ($p<0.05$ with correction for false discovery rates). Furthermore, posthoc testing showed that scores for PC1 were higher for 16% sucrose compared to 2% and 4% sucrose. In contrast to the results for PC1, scores for PC2 only differed at the extremes of the sucrose concentrations, i.e. sessions with 0% or 2% sucrose versus sessions with 16% sucrose.

Intermediate Summary: Measures of Value in the Progressive Ratio Licking Task

Together, the multivariate analyses suggest that sucrose concentration impacts a set of correlated behavioral measures in the progressive ratio licking task. This set of measures includes the classic “motivational” measure of breakpoint as well as several related instrumental (time to break and total licks) and consummatory (bout number and percent of time in bouts) measures. This analysis framework was used to evaluate the effects of reversibly inactivating the medial frontal cortex in Experiment 2.

Experiment 2: Effects of Reversible Inactivation of the Medial Frontal Cortex

Experiment 1 determined which of the various behavioral measures from the progressive ratio licking task are sensitive to value (0-16% liquid sucrose). We carried out Experiment 2 to evaluate the roles of various parts of the medial frontal cortex in progressive ratio performance using pharmacological reversible inactivation methods. Twenty rats (17 males and 3 females)

were tested. The inclusion of only 3 females did not allow us to evaluate effects by sex. As in Experiment 1, the mass of the males and females was quite different (400-480g for males and 250-300g for females).

Cannulae placement is shown in Figure 4. Infusion cannulae were implanted along the rostral-caudal axis of the medial frontal cortex, including the prelimbic cortex (+3.2 to +3.7 AP, N=6), caudal medial orbital cortex (+4.2 AP, N=7), rostral medial orbital cortex (+4.7 AP, N=5), and the medial portion of frontal pole (+5.2 AP, N=2). Notably, the two rats with implants in the frontal pole were misplaced, as the surgeries had targeted the rostral medial orbital cortex. Surgery on this part of the medial frontal cortex was a challenge. Only 8 of 15 rats that received implants targeting the rostral medial orbital cortex survived the surgeries. (Success rates for the more caudal medial frontal cortex in this study and others in our lab has been near 80%.) Several of these animals died several days after experiencing a normal recovery from surgery. One of these rats was excluded from testing due to obvious clogging of the injection cannulae.

Cortical inactivation, without regard to the precise location of the inactivation, had a significant effect over all behavioral measures (Figure 5; Multivariate ANOVA – $F(1,15) = 36.818$, permutation $p=0.0000$). At the univariate level, response rate was the only behavioral measure to show sensitivity to inactivation of the medial frontal cortex (paired permutation t-test: $t(1,17)=-3.097$, $p=0.00568$). All other measures were insensitive to inactivation of the various parts of the medial frontal cortex. The two rats with cannulae in the frontal pole were excluded from this and all other statistical analyses.

Data from the two rats with cannulae in the medial part of the frontal pole are depicted by gray dashed lines. These two rats were outliers across all measures compared to the other 18 animals. Their instrumental engagement (total licks, breakpoints, time to break, and response

rate) was very low following the infusions of muscimol (Figure 5A-D). In fact, these measures were below those reported for the rats in Experiment 1 when tested with water (horizontal dotted lines). In a similar way, the two frontal pole rats showed very long mean inter-lick intervals (low licking frequencies – Figure 5E), emitted very brief licking bouts (Figure 5F), emitted very few licking bouts (Figure 5G), and engaged in licking for less than 10% of the experimental sessions (Figure 5H). These animals were not considered further in the present study due to the non-specific nature of behavioral impairments found after inactivation of the medial frontal pole.

Univariate ANOVAs were used to assess the impact of the location of the cortical inactivation within the prelimbic and medial orbital areas on the collection of behavioral measures. The effects of inactivation on response rate depended on where the inactivation was made (Figure 6E; $F(2,15)=7.951$, $p=0.0045$). Posthoc comparisons ($p<0.05$) showed evidence for this effect being driven by rate increases following prelimbic inactivation and rate decreases following inactivation of the rostral medial orbital cortex. The slope of the robustly fitted regression line to these data (not shown) was significantly offset from 0 (slope: -0.22270 , 95% CI: -0.36592 to -0.07791).

A second effect of inactivation was to increase the time to the breakpoint for rats with inactivations of the medial orbital cortex compared to those with inactivations of the prelimbic cortex (Figure 6D; $F(2,15)=8.143$, $p=0.005$; posthoc comparisons: $p<0.05$ for PL vs rostral and caudal mOFC, but not for the comparison between rostral and caudal mOFC). The slope of the robustly fitted regression line to these data (not shown) was significantly offset from 0 (slope: 0.44318 , 95% CI: 0.16000 to 0.72030).

No other measures of performance, including the breakpoint ($F(2,15)=1.066$, $p=0.392$), were altered by inactivation of the pregenual medial frontal cortex caudal to the rostral pole.

Scores for the principal components, based on the same measures from Experiment 1, were not sensitive to cortical inactivation (PC1: $F(2,15)=1.718$, $p=0.213$; PC2: $F(2,15)=2.899$, $p=0.0862$).

Discussion

Summary of findings

The goal of this study was to determine the role of medial frontal regions (prelimbic and medial orbital) in the control of progressive ratio performance using a licking-based task (Sclafani & Ackroff, 2003). The task differs from traditional progressive ratio procedures as it does not require the animals to do anything more than stand at a lickometer and repeatedly lick. As a result, the procedure does not depend on arbitrary skills such as making rapid sequences of lever presses or nose pokes and does not require the rats to move between response and reward ports. Given the relatively limited use of the progressive ratio licking procedure, it was first necessary to determine which behavioral measures of progressive ratio responding and which microstructural aspects of licking are sensitive to reward value by manipulating sucrose concentration. Multivariate analyses revealed that a set of behavioral measures strongly covaried with value (the percent of time in licking bouts, breakpoint ratio, total licks, number of licking bouts, and time to break).

After working out which behavioral measures are sensitive to value, we then used reversible inactivation methods to determine the roles of the medial orbital and prelimbic areas of the MFC in the control of progressive ratio licking. As in some previous studies on medial frontal regions using lever-press versions of progressive ratio performance (Kheramin et al., 2005; Schweimer & Hauber, 2005; mice lesioned after training in Gourley et al., 2010), we found no effects of reversible inactivation on breakpoint or most other measures associated with

value when the rostral medial orbital or caudal prelimbic cortices were inactivated across 18 rats (Figures 5B and 6B). An exception was the time to break (Figure 6D), which is discussed below. Changes in breakpoint are commonly interpreted as evidence for changes in the “reward strength”, or the efficacy of reinforcement to maintain responding under high response ratios (Hodos, 1961), and has commonly been interpreted as “motivation” (Arnold & Roberts, 1997). Based on these interpretations, our data suggests that the medial orbital and prelimbic areas do not mediate motivational control over progressive ratio responding in the progressive ratio licking task.

Effects on breakpoints

For the inactivations in the medial orbital cortex, we had expected to find reductions in breakpoint, similar to Gardner et al. (2018). This outcome was expected based on our previous studies on the medial orbital region in the control of consummatory licking (Amarante et al., 2017; Parent et al., 2015a; Parent et al., 2015b), which demonstrate that the medial orbital cortex has an enabling or excitatory role in maintaining licking behavior. The alternative, that breakpoints would elevate, has been reported in two studies (mice lesioned before training in Gourley et al., 2010; Münster & Hauber, 2017), and would indicate that the medial orbital cortex has an inhibitory role in the control of progressive ratio performance.

Inhibition is a classic interpretation of orbitofrontal function (Dias et al., 1996). However, more recent studies have emphasized a role for the medial orbital areas in predictions and evaluations of behavioral outcomes (Rudebeck & Murray, 2014; Rudebeck et al., 2017) and inferences based on learned associations between actions and outcomes (Bradfield et al., 2015). As such, disruptions of medial orbital control should reduce, not increase, breakpoints, as is also

predicted based on studies of the medial orbital cortex in incentive contrast procedures (e.g. Parent et al., 2015a). If we had found elevated breakpoints in our study, then a reasonable interpretation might have been that the medial orbital cortex controls a shift from inhibition of competing actions during instrumental licking to release of consummatory behavior when rewarding fluids become available. However, we did not find evidence for this interpretation of medial orbital function.

Differences in the outcomes of published studies on medial frontal areas in progressive ratio procedures (McGregor et al., 1996; Kheramin et al., 2005; Schweimer & Hauber, 2005; Gourley et al., 2010; Gourley et al., 2016; Münster & Hauber, 2017; Gardner et al., 2018) could be due to a number of factors. These include differences in species (rats vs mice), precise location of the lesion or inactivation (dorsal vs ventral portions of the orbital cortex), behavioral history (exposed to progressive ratio alone or used in other studies prior to progressive ratio), extent of training (days vs weeks of experience), type of reinforcement (food, liquid sucrose, drugs), level of food regulation (80-92% free access weights), proximity to reward (adjacent reward port or port on opposite wall of behavioral chamber), presence or absence of Pavlovian cues signaling reward delivery, and the method, spatial extent, and cell type affected by the brain perturbations (lesion, muscimol, chemogenetics, optogenetics).

Our findings on breakpoint are similar to three published studies. Kheramin et al. (2005), Schweimer & Hauber (2005), and Gourley et al. (2010) found no effects of lesions in three different cortical areas, two different actions, two different species, and very different levels of training. The Kheramin study used rats, required lever pressing, and lesioned the ventral orbital area after 60 training sessions. The Schweimer study also used rats, required lever pressing, and lesioned the perigenual prelimbic and cingulate after 6 training sessions. The Gourley study used

487 mice, required nose poking, and lesioned the prelimbic and medial orbital areas after 5 training
488 sessions.

489 Gourley et al. (2010) also examined effects of lesions made before training (5 sessions),
490 and found clear and differential effects of lesions in the prelimbic and medial orbital areas on
491 breakpoints. Breakpoints decreased with prelimbic lesions and increased with medial orbital
492 lesions. A subsequent study by Gourley et al. (2016) used a chemogenetic method and other
493 molecular tools that stimulated the medial orbital region and found reductions in breakpoints in
494 task naive animals. A similar finding was reported by Münster & Hauber (2017) for rats that
495 received medial orbital lesions prior to training (their experiment 1). The implications of the
496 studies are that medial frontal areas do not control breakpoint in experienced animals, but are
497 crucial for learning to perform the procedure.

498 However, other findings in the Münster & Hauber (2017) complicate this interpretation,
499 and do not agree with the results of our study for the medial orbital cortex. Münster & Hauber
500 (2017) also examined effects of pharmacological inactivation (muscimol and baclofen) and
501 excitation (picrotoxin) in the medial orbital cortex. The studies used rats with just 3 days of
502 training prior to the pharmacological testing. Similar to the effects of lesions, reversible
503 inactivation increased breakpoints and reversible excitation decreased breakpoints.

504 A major difference between our study and the one by Münster & Hauber (2017) was the
505 extent of training prior to reversible inactivation. Münster & Hauber (2017) trained rats for 3
506 sessions prior to pharmacological testing. By contrast, the rats in our study were trained for
507 many more sessions (median of 14 sessions; range 3 to 35). Variable training occurred for two
508 reasons. First, we trained animals until their breakpoints were asymptotic (see Methods), and
509 different rats need more or less training to reach stable breakpoints. Second, some of the rats

were used in Experiment 1 prior to the inactivation study. Permutation ANOVA found no evidence for variability in training impacting breakpoint ($F(1,16)=0.6293$, $p=0.44$), time to breakpoint ($F(1,16)=0.0053$, $p=0.945$), and response rate ($F(1,16)=1.01$, $p=0.328$).

Two other studies further complicate understanding the role of medial frontal regions in progressive ratio performance. McGregor et al. (1996) made dopamine depletions in the region of the prelimbic cortex (anatomy not shown). They reported increased breakpoints in rats working for cocaine, a finding that is not in line with the findings of Schweimer & Hauber (2005) and Gourley et al. (2010). Breakpoints for cocaine were relatively low (<15) compared to those for food or sucrose pellets in the Schweimer (>40) and Gourley (>60) studies. The difference in reinforcer (drug vs food) and range of breakpoints might explain the different findings among these studies.

Finally, a recent study by Gardner et al. (2018) reported *reduced* breakpoints following optogenetic inactivation of the ventral part of the medial orbital cortex of rats. The rats had experience in an operant choice task, which failed to find effects of medial orbital inactivation, and then were trained over 2 sessions to lever press and then to perform a progressive ratio design over 4 additional sessions. The range of breakpoints was low in this study (<20) in comparison to the lever-press studies in rats by Schweimer & Hauber (2005) and Münster & Hauber (2017).

Effects on response rate and time-to-break

While we found no effects of medial frontal inactivation on breakpoints, it was obvious to the experimenters during the inactivation test sessions that some of the rats finished the sessions earlier or later than expected based on their performance history and control sessions.

While many previous studies have examined effects of drugs (Blasio et al., 2014; Richardson & Roberts, 1996), lesions (Eagle et al., 1999), and inactivations (Zhang et al., 2003) of the striatum, and detailed mathematical models of progressive ratio performance have been developed (Bizo & Killeen, 1997), no previous study to our knowledge has reported effects of any experimental perturbations on the time to break. Statistical analysis of the time to the breakpoint, which included a factor for the locations of the muscimol infusions (Figure 6D), confirmed our impression that inactivation affected the time taken to complete the task. This analysis revealed evidence for a significant effect of cortical inactivation. In addition, a more dramatic effect was found for the effect of inactivation on the response rate, which increased over all animals (Figure 5D) and diverged between infusions into the prelimbic and rostral medial orbital cortices (Figure 6E). These findings suggest that the rostral and caudal halves of the medial frontal cortex have reciprocal roles in controlling the pace of progressive ratio performance.

Several previous studies suggest a role of the medial frontal cortex in the control of behavioral pace (humans: Stuss et al., 2005; non-human primates: Chen et al., 1995; rats: Horst & Laubach, 2009). A neural recording study found evidence for an encoding of pace by a subpopulation of medial frontal neurons in rats performing a delayed alternation task (Horst & Laubach, 2012). Other studies have reported issues with temporal control in maintaining responses over delay periods (Narayanan et al., 2006; Risterucci et al., 2003) and with performing interval timing tasks (Parker et al., 2013). These findings might reflect a more general role of the medial frontal cortex in tracking goal-directed actions, especially the time between behavioral events such as lever presses and reward delivery (Bekolay et al., 2014).

From this perspective, the role of the MFC in progressive ratio performance might be to maintain behavioral engagement, i.e. to keep animals “on task”.

Neural circuits for the control of engagement by the medial frontal cortex

There was no effect of sucrose concentration on response rate, suggesting that changes in response rate following inactivation are independent from changes in reward value. Distinct anatomical circuits from the prelimbic and medial orbital cortices might mediate the diverging control that these cortical regions have over behavioral pace. Tract-tracing studies have established that the rostral and caudal halves of the rodent MFC are quite distinct with regards to projections to the hypothalamus (Floyd et al., 2001), periaqueductal gray (Floyd et al., 2000), striatum (Berendse et al., 1992; Bradfield et al., 2018), and amygdala (Gabbott et al., 2005). Neurons in the medial orbital (rostral) region project to the ventral striatum (Bradfield et al., 2018; Gabbott et al., 2005) and to parts of the lateral hypothalamus and periaqueductal gray (Floyd et al., 2001) that are associated with decreased behavioral activation and the control of autonomic and homeostatic processes (Bandler et al., 2000), suggesting that this circuit plays a role in the maintenance of feeding behavior. This region is a core component of the “medial network” of rodents, primates, and humans as described by Ongür & Price (2000). By contrast, neurons in the prelimbic (caudal) region project to the dorsal striatum (Gabbott et al., 2005) and parts of the lateral hypothalamus and periaqueductal gray (Floyd et al., 2001) that are associated with increased behavioral activation and emotional “fight-or-flight” responses (Bandler et al., 2000). This area may be homologous to the pregenual anterior cingulate cortex of primates including humans (Laubach et al., 2018). Removing reciprocal control by these two

577 halves of the medial frontal cortex over these subcortical systems may have resulted in changes
578 in how quickly animals completed the progressive ratio task.

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582

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584 and wrote the manuscript. Hannah Goldbach performed the experiments. Mark Laubach
585 designed and performed the experiments, assisted with data analysis, and wrote the manuscript.

586

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Figure 1. Effects of sucrose concentration on instrumental measures in the progressive ratio licking task. (A) Diagram of the behavioral chamber and experiment design. Rats were tested across increasing concentrations of sucrose solution, from 0% (water) to 16% sucrose, interleaved with sessions of 16%. Only the final presentation of 16% sucrose was included in the analysis. (B) Depiction of instrumental measures from an example session with 16% sucrose rewards. Licks are plotted over experimental time. Completion of each ratio produced reward at the timings indicated by vertical gray lines. (C) Licks per session increased with sucrose concentration. Rats licked less for water compared to all levels of sucrose and more for 16% sucrose compared to 2% and 4% sucrose. (D) Breakpoint increased with sucrose concentration. Breakpoint was lower for water compared to all levels of sucrose and higher for 16% sucrose compared to 2% and 4% sucrose. (E) Time to break denoted the presence of sucrose. Rats spent more time engaged in the task when they consumed any level of sucrose compared to when they consumed water. (F) Response rate was not sensitive to sucrose concentration. In panels C-F, black dots indicate measures for each of the 22 rats, thick gray lines indicate the mean measure for each sucrose concentration, and significant differences ($p < 0.05$ from the permutation posthoc tests with adjustments for false discovery rates) are denoted by bars and lines.

Figure 2. Effects of sucrose concentration on consummatory measures in the progressive ratio licking task. (A) Definitions of “microstructural” measures of licking. Two of these measures were highly sensitive to sucrose concentration. The number of licking bouts (E) increased with sucrose concentration. Fewer bouts were emitted for water compared to all levels of sucrose. More bouts were emitted for 16% sucrose compared to 2% and 4% sucrose. The percent of time spent licking (F) increased with sucrose concentration. Rats spent less time consuming water compared to all levels of sucrose. More time was spent licking for 16% sucrose compared to 2% and 4% sucrose and for 8% sucrose compared to 2% sucrose. There was no effect of sucrose on interlick interval (B) or bout duration (D). The coefficient of variation (C) for inter-lick interval was significantly higher for water compared to all levels of sucrose. In panels B-F, black dots indicate measures for each of the 22 rats, thick gray lines indicate the mean measure for each sucrose concentration, and significant differences ($p < 0.05$ from the permutation posthoc tests) are denoted by bars and lines.

Figure 3. Multivariate analysis of instrumental and consummatory measures. (A) Correlation matrix for 9 behavioral measures, with functional groupings identified using hierarchical cluster analysis. The strength of the correlation is represented by the size of the squares associated with each variable and the sign of the correlation is indicated by color (black=positive, white=negative). The autocorrelation across animals is plotted on the main diagonal of the matrix (northwest to southeast). Black lines around the set of variables denote the three clusterings identified by the hierarchical cluster analysis. (B) F statistics from univariate ANOVAs for the effect of sucrose concentration on each of the behavioral measures. (C) Scree plot of eigenvalues from a principal component analysis of the collection of behavioral measures. (D) Eigenvectors for the behavioral measures over the 3 principal components after varimax rotation. The upper plot shows the raw eigenvectors and the lower plot shows the eigenvectors weighted by the fraction of variance. As in panel A, the size of the eigenvectors is denoted by the size of the plotted squares and the sign of the eigenvectors is denoted by color. (E) Scores for the first principal component (PC1) were sensitive to sucrose concentration. Scores were lower for water compared to all levels of sucrose. Scores were higher for 16% sucrose compared to 2% and 4% sucrose. (F) Scores for the second principal component (PC2) were not sensitive to sucrose concentration.

Figure 4. Cannula placement. Infusion cannulae were implanted into the prelimbic and medial orbital areas, spanning from +5.2mm to +3.2mm anterior to bregma, in 20 rats. Cannula tips are denoted by black dots. Group sizes by AP: +5.2, n=2; +4.7, n=5; +4.2, n=7; +3.7, n=4; +3.2, n=2. Given similarity across all behavioral measures for rats with cannulae at +3.7 and +3.2 AP, data from these rats was merged for the analyses reported in this manuscript. This region of the cortex was denoted as “PL” for the prelimbic cortex (n=6). Rats with cannulae centered at +4.2 AP were denoted as “caudal MO” (n=7) and rats with cannulae centered at +4.7 AP were denoted as “rostral MO” (n=5). The two rats with cannulae at +5.2 AP were denoted as “frontal pole”.

Figure 5. Effects of inactivation on behavioral measures associated with reward value.

Inactivation selectively increased the animals' response rate (panel D, paired t-test: -3.097 , $p=0.00568$). Inactivation had no effect on the other behavioral measures. The precise anatomical locations of the infusions are mostly ignored in these plots, with the exception of data from the two rats with cannulae placed in the rostral pole ($+5.2$ AP). Data from these rats are shown using gray dashed lines and were excluded from statistical analysis. This was done because most behavioral measures from these rats were below those associated with performing the task for water (Experiment 1; horizontal black dashed lines). An exception was the inter-lick intervals (E), which were exceptionally long for these two rats. Results are not shown for the coefficient of variation for the inter-lick intervals, as it was nearly identical to that shown for the average inter-lick intervals.

Figure 6. Effects of inactivation as a function of location within the medial frontal cortex.

Inactivation had no effect on most measures. However, the time to break (D) decreased following inactivation of PL and increased following inactivation of rostral mOFC. Inactivation also altered the response rate (E), which increased following inactivation of PL and decreased following inactivation of rostral mOFC. Inactivation had no effect on the number of licking bouts per session (C), although there was a trend for increased numbers of bouts for rats with inactivation centered on the rostral mOFC. In all panels, black dots indicate normalized measures for each of the 18 rats (see Methods), thick gray lines indicate the mean measure for anatomical grouping, and significant differences ($p < 0.05$ from the permutation posthoc tests) are denoted by bars and lines.