UNIVERSITY OF CALIFORNIA SAN DIEGO

Orbitofrontal cortex projecting mediodorsal thalamic population's contributions to instrumental and incentive learning and performance

A dissertation submitted in partial satisfaction of the
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by

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The dissertation of Ege Ayse Yalcinbas is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I would like to dedicate this dissertation to the future generations of neuroscientists who will attain a more complete understanding of higher-order thalamic regions and their roles in the instantiation of perception and cognition.

EPIGRAPH

Deep ignorance, when properly handled, is also superb opportunity. The right question is intellectually superior to finding the right answer.

-E. O. Wilson, *Letters to a Young Scientist, pg. 177*

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ABSTRACT OF THE DISSERATION

Orbitofrontal cortex projecting mediodorsal thalamic population's contributions to instrumental and incentive learning and performance

by

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Cognitive control processes do not solely rely on the prefrontal cortex (PFC) proper. Mediodorsal thalamus (MD), the higher-order thalamic region known to be prominently connected with the PFC, has been recognized as an important node in the cortico-striatal-thalamic-cortical loops mediating flexible goal-directed behavior, both in clinical and basic research. Previously, studies trying to understand MD activity and function have largely examined or manipulated the structure as a whole. Recently, there has been an increasing appreciation of, and methods to target, the subpopulations within

the MD as they relate to the functions of their respective PFC targets; but most *in vivo* studies focus on MD projections into medial and dorsal PFC. The lateral orbitofrontal cortex (lOFC) also receives input from the MD, and has been implicated in various aspects of goal-directed decision-making such as outcome valuation and maintaining an up-to-date internal representation of tasks and the contingencies therein; yet MD's contributions to lOFC functions have remained unclear.

In this dissertation, I sought to image and manipulate the endogenous activity pattern of the MD projection population into lOFC during the learning and performance of a self-initiated goal-directed task, which to our knowledge is the first time this MD-PFC subcircuit has been examined in this manner *in vivo*. We found that the activity of the MD terminal population in lOFC was differentially sensitive to trials based on the probabilistic outcome of instrumental actions. In concert, we found that animals' expectation built across learning and changing task requirements, with expectation-modulated instrumental performance affected by optogenetically inhibiting the activity of lOFC projecting MD somas. We did not find evidence of motivational state-induced outcome value representation in the MD-lOFC terminal population; however, attenuating the activity of MD-lOFC projection neurons during outcome revaluation and, in particular, the use of updated outcome value in extinction did compromise adaptive instrumental behavior. These findings suggest that MD input population into lOFC provides prospective information that modulates instrumental actions within the overall cognitive control framework of monitoring interactions with the world, comparing expectations with actual experiences, and adapting an internal model of the world in order to optimize goal-directed behaviors.

INTRODUCTION

A cortical region of interest: Orbitofrontal cortex

The orbitofrontal cortex (OFC) has emerged as a cortical region of interest for understanding learning and decision-making – both in its flexible, adaptive forms as well as pathological forms such as addiction or obsessive compulsive disorder (Ragozzino, 2007, Fettes et al., 2017). From an anatomical and functional perspective, the OFC is considered to have two separable subregions along the medial-lateral axis (Rudebeck and Murray, 2011). Lateral orbitofrontal cortex (lOFC) neurons have been found to encode outcome-related cues or responses, outcome expectancy across instrumental learning, and subjective value of biologically relevant outcomes, among other decision variables (Schoenbaum et al., 1998, Ichihara-Takeda & Funahashi, 2006, Feierstein et al., 2006, Padoa-Schioppa & Assad, 2006, Wallis, 2011, Moorman & Aston-Jones, 2014, Izquierdo, 2017). These and other findings pertaining to lOFC function implicate the region in forming associations between external stimuli, responses/actions, and outcomes – and relatedly, building and updating accurate predictions based on the consequences of behaviors (Takahashi et al., 2011). Many lOFC-related findings could be subsumed under the more recently proposed cognitive map theory, whereby lOFC is thought to be involved in adapting an internal associative structure that takes into account observable and latent variables to guide goal-directed behavior within a task space (Stalnaker et al, 2015, Behrens et al., 2018). Being embedded in a broader cortico-striatal-thalamic-cortical loop, lOFC clearly does not function alone to accomplish these feats; however, the OFC field has largely centered on the activity of OFC itself to

understand its function (though there has been fairly extensive focus on its connectivity with the amygdala in particular). Considering OFC's prominent reciprocal connectivity with the mediodorsal thalamus (MD), how MD afferents coming into lOFC may be contributing to the variety of functions lOFC has been implicated in has remained underexplored.

Mediodorsal thalamus as a prominent synaptic partner of the prefrontal cortex

The prefrontal cortex (PFC) is thought to underlie the instantiation of cognitive control in organisms that possess it (Miller, 2000). Historically, the observation that the MD has a striking topographic connectivity with the OFC and other PFC regions has provided an anatomical framework within which to define and study the PFC across many different species (Rose & Woolsey, 1948, Ray & Price, 1992 and 1993, Xu et al., 2021). The attention that MD's PFC projections have garnered is in no small part due to mounting evidence that higher-order thalamic nuclei like the MD which are primarily driven by deep layers of cortex are involved in more integrative and complex cortical processing than first-order thalamic nuclei which are largely thought of as faithful relays of sensory information from peripheral systems (Mukherjee et al., 2020). Mostly based on investigations of MD's projections into dorsal and medial PFC, putative functions of MD have been interpreted to be sustaining cortical activity, modulating synchrony between cortical regions to facilitate cortico-cortical information transfer, resolving input uncertainty, and switching between cortical representations based on task context (Saalmann, 2014, Acsady, 2017, Rikhye et al., 2018, Mukherjee et al., 2021). Of clinical importance, alterations in MD-PFC circuitry have been linked to multifaceted

neuropsychiatric conditions such as schizophrenia and autism spectrum disorders (Ouhaz et al., 2018). Other human studies have shown that behavioral phenotypes following MD damage mirror executive function deficits observed in patients with PFC damage (e.g. set shifting, attention, working memory deficits), though in these studies, the extent of MD damage is often not contained to a rigorously defined subregion (Van der Werf et al., 2003, Pergola & Suchan, 2013).

Gross-level lesion studies are not ideal for a nuanced exploration of MD function given that we know MD subregions can be distinguished on the basis of myeloarchitecture, cytoarchitecture, and topographic PFC connectivity (Ray & Price, 1993, Alcaraz et al., 2016). There are also more recent reports of genetically *and* anatomically distinct MD populations that tend to synapse onto different inhibitory cell types enriched in different layers of the PFC (Mukherjee et al., 2021). Thus, it is not surprising that even though MD is referred to as a singular thalamic nucleus, mounting evidence from across the thalamus is revealing that a given thalamic 'nucleus' is not functionally homogeneous as a result of its constituent cells' varied input-output connections (Rikhye et al., 2018, Mukherjee et al., 2021). Historically however, notable studies looking into MD function have taken blunt approaches like excitotoxic lesions. With such lesion studies, in addition to not being able to target particular MD subregions or populations, there is the question of when the lesion surgeries were conducted relative to task training and the potential implications this could have on experimental results and interpretation.

One set of studies in which MD was bilaterally lesioned in rats *before* training showed insensitivity to outcome devaluation if tests were conducted in extinction, as well

as insensitivity to contingency degradation even though the rats' ability to differentiate between the two actions and their respective outcomes was determined to be intact (Corbit et al., 2003). Thus, MD was concluded to be important for encoding instrumental action-outcome associations, and utilizing it for appropriate response selection. In contrast, *post-training* excitotoxic lesions of the MD did not affect sensory-specific outcome devaluation but did impair appropriate cue-modulated action selection (pavlovian-instrumental transfer test) and appropriate conditioned approach behavior following cue-outcome contingency degradation (Ostlund & Balleine, 2008). Apart from the difference in when MD was irreversibly taken offline in these experiments, these conflicting results concerning MD's role in instrumental behavior are hard to reconcile without accounting for the number and types of behavioral tests, the order in which they were conducted, and without knowing the extent to which MD's various subregions were damaged, as different types of associative learning and behavior are thought to be mediated by different neural substrates (Bouton et al., 2021). Critically, there is abundant evidence that compensatory changes can occur in the broader network as a consequence of lesions, which makes it difficult to interpret null results from particular behavioral tests (Reuss et al., 2020, Adam et al., 2021). Granted, however, transient pharmacological inhibition of MD has also yielded similar results to the post-training MD lesion studies in terms of implicating MD in adapting to changes in action-outcome contingencies and cue-modulated action selection (Parnaudeau et al., 2015). Ultimately, in order to begin to achieve a more nuanced understanding of the role that MD plays in cognition (which is a very broad and often unhelpful term), there is a need to approach gross-level manipulations of MD with caution, and move towards investigating MD subcircuits'

endogenous activity patterns in the context of their connectivity as well as during particular behaviors thought to be mediated by their particular cortical projection fields. In general, there is a dearth of knowledge on the activity of certain MD subregions in relation to their cortical synaptic partners. As mentioned before, one such underexplored subcircuit is the MD population that projects to the OFC.

What is known about the MD-lOFC subcircuit?

Most of the exploration of MD activity *in vivo* has been focused on MD projections into medial PFC (Bolkan et al., 2017, Schmitt et al., 2017, Rikhye et al., 2018). Less attention has been given to the functional role of its medial-central (MDm/c) projections into lOFC even though primates and nonprimate mammals share homologs of agranular OFC-insular cortex, whereas homology is more contentious for other regions of PFC (Preuss & Wise, 2021). From a clinical perspective, diffusion tractography studies in patients with schizophrenia have demonstrated reduced thalamo-orbitofrontal connectivity which positively correlates with reduced cortical thickness in the lOFC and frontal polar cortex (Kubota et al., 2013). Such findings further motivate the need for a better understanding of the activity of the MD-OFC subcircuit *in vivo* in both pathological and non-pathological contexts, for it would clearly be beneficial to understand the functional implications of compromised MD-lOFC anatomical connectivity.

A substantial portion of *in vivo* studies on the MD-OFC subcircuit is disconnection studies. One such crossed disconnection study lesioned MD in primates that already had amygdala and OFC lesions, and conducted a sensory-specific outcome devaluation paradigm (Izquierdo & Murray, 2010). Similar to the MD lesion studies in rats (Corbit et

al, 2003), the functional disconnection results suggested an impairment in appropriately updating object-value associations or the ability to use this information to guide appropriate action selection. However, another study that disconnected OFC from MD did not find an impairment in sensory-specific outcome devaluation or updating action-outcome contingencies (Fresno et al., 2019). With crossed disconnection studies, even though the approach is more circuit-specific than just lesioning MD proper, the potential caveat to consider is that while thalamocortical projections are known to be almost exclusively ipsilateral, returning corticothalamic pathways can cross to the other hemisphere. Thus, it is difficult to claim that MD-OFC functional connectivity is completely severed using this approach. Additionally, with the crossed disconnection approach, could other MD-PFC subcircuits compensate for compromised MD-OFC functional connectivity? For instance, a recent study found histological evidence that the MD-OFC subcircuit may be compensating for the loss of the prelimbic cortex during the training of a task guided by both action-outcome and cue-outcome associations (Fisher et al., 2020 and 2021). This potential caveat could be occluding functional effects of an MD-OFC crossed disconnection in behavioral tests that appear to show no deficit.

In the wake of these circuit-specific disconnection approaches, we are still left with the fundamental question of precisely *when* MD-OFC projections are recruited *in vivo* under normal circumstances, without permanent damage to the broader circuit. There are very few *in vivo* studies that performed temporally specific reversible manipulation of the MD-OFC pathway to test its involvement in a particular task or behavior. In one experiment of a larger study, MD terminals in lOFC were inhibited during the performance of a delayed non-match to sample task; the results suggested that this subcircuit isn't

critical for working memory in mice (Bolkan et al., 2017). Another recent study optically induced plasticity in the MD-OFC pathway to test whether OFC activity and stress-induced deficits in reversal learning could be bidirectionally modulated by MD input (Adler et al., 2020). Much like the disconnection studies, these experiments provide very general insight into whether the MD-OFC pathway's activity during tasks of a particular category (e.g. working memory, flexible decision-making) are critical for or a contributor to performance, but without the caveat of permanent damage to the circuit as in the case of disconnections. However, what particular aspects of the external world (i.e. task context) or internal state of the animal recruit the MD-OFC pathway remain unknown.

Thus far, insights into unperturbed endogenous activity have been limited to recordings from MD proper, which - as I have established in previous sections - contains different populations within it. One such study recorded local field potentials from OFC and piriform cortex, and separately recorded single units as well as LFPs from the MD in a two-alternative olfactory discrimination task (Courtiol & Wilson, 2016). Some notable findings were MD unit activity modulation during the pre-sampling period when the animals were anticipating the presentation of the stimulus, and differential firing during the pre-goal port approach period depending on the choice. Similar to other recording studies in the OFC, MD units were interpreted to encode spatio-motor aspects of the goal during decision-making (Feierstein et al., 2006). Perhaps of greatest interest given its pertinence for understanding subcircuit dynamics, this study uncovered MD spike phase locking to LFP beta oscillations recorded in OFC during goal port approach. Even though these are findings within the scope of an olfactory task, they are some of the few insights we have gleaned into unperturbed activity dynamics in the MD-OFC subcircuit which,

generally speaking, seems to be engaged during learning and flexible goal-directed decision-making. There are computational methods to identify directionality in interregional oscillatory dynamics, but perhaps a more straightforward way to isolate MD's real-time contribution to OFC function is to record from the MD projection population coming into lOFC. This gap in knowledge is the starting point for the set of studies described in this dissertation.

Summary

Throughout the decades-long journey of trying to understand how the prefrontal cortex endows organisms with various cognitive capabilities, there has only recently been an intense focus on investigating the function of the PFC through the lens of its thalamic synaptic partner, the mediodorsal thalamus. Indeed, many new exciting ideas regarding the functional role of higher-order thalamic nuclei in cognition have emerged from studying MD's projections into dorsal and medial PFC regions. Of interest here are the prominent MD projections into lateral orbitofrontal cortex; specifically, what types of information this population may be carrying or what kinds of modulatory or other functional influence this input may be having on the local microcircuit within lOFC.

Investigations into the function(s) of the OFC have historically been rather focused on task-related activity of the region proper, but there is much to be gained by examining how long-range inputs such as from the MD may be influencing the rich array of local circuit components in the lOFC (Chapter 1). Until recently, imaging techniques to investigate long-range projection activity *in vivo* in freely behaving animals were limited, but the genetic tractability of mice and current viral tools available for targeting neural

populations with projection specificity make the set of studies described in this dissertation possible. I leverage recently developed axon-enriched optical tools to image the population activity of MD terminals in lOFC and answer the question: What aspects of an instrumental task structure recruit the activity of the MD population that projects to the lOFC, a region implicated in updating cognitive maps and flexible goal-directed decision-making? Relatedly, I address whether this recruitment is modulated by relevant task parameters, if at all, and whether inhibition of this subcircuit during epochs of differential recruitment affects task performance (Chapter 2). To our knowledge, these experiments are the first reported instance of imaging and manipulating the endogenous activity patterns of MD projections into lOFC during the learning and performance of a self-initiated instrumental task. By employing this strategy, I thus demonstrate for the first time that the activity of the MD terminal population in the lOFC reflects outcome expectancy as informed by experience with the instrumental task contingency under probabilistic control. Inhibiting MD somas projecting to lOFC reveal a causal link between this activity and expectation-modulated behavioral performance. Within a forward model framework, these findings suggest a role for the MD-lOFC subcircuit in adapting and updating an internal model for a goal-directed task, which ultimately serves to improve cortical predictions and optimize task performance.

While Chapter 2 focuses on MD-lOFC subcircuit activity dynamics pertaining to instrumental contingencies between actions and outcomes, I wanted to next ask whether another hallmark of goal-directed behavior - that is, the ability to update outcome value representation and adapt behavior accordingly - would be reflected in this subcircuit (Chapter 3). The standard way to assess the outcome dependency of an instrumental

action is to conduct an outcome devaluation test, which can be performed in multiple ways. In this case, I make use of motivational state-dependent incentive learning, a process by which the motivational state of animals is shifted through satiation and the animals are subsequently re-exposed to the instrumental outcome in their new motivational state in order to update their outcome value representation. Our lab has shown that intact OFC function is important during incentive learning in order to observe appropriate adjustment of instrumental seeking action on an extinction test day (Baltz et al., 2018). In Chapter 3 I describe experiments in which I use the aforementioned genetic and imaging tools to examine what role the MD-lOFC subcircuit may be playing in this process. I find that the MD terminal population in lOFC is recruited during outcome consumption in a re-exposure session following a motivational shift as well as no shift. The population activity does not seem to be reflective of a change in outcome value. Chemogenetically attenuating lOFC projecting MD neurons' activity during outcome re-exposure following a motivational shift affects adaptive instrumental performance on a subsequent extinction test compared to control groups, and even more starkly so if the attenuation is conducted on test day. Thus, the results of experiments described in Chapter 3 suggest that the MD-lOFC subcircuit is likely not as involved in outcome representation itself as much as adjusting instrumental behavior based on an updated internal model of the task that takes into account the changed outcome value.

Collectively these chapters address a gap in our knowledge and approach to understanding OFC function, and further pave the way for more granular investigation of MD activity and function that takes into account subcircuit specificity. This finer level of

understanding will likely have critical implications for treating multi-faceted psychiatric disorders that present with debilitating cognitive symptoms.

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CHAPTER ONE: Call for a more balanced approach to understanding orbital frontal cortex function

Abstract

Orbital frontal cortex (OFC) research has historically emphasized the function of this associative cortical area within top-down theoretical frameworks. This approach has largely focused on mapping OFC activity onto human-defined psychological or cognitive constructs and has often led to OFC circuitry bearing the weight of entire theoretical frameworks. New techniques and tools developed in the last decade have made it possible to revisit long-standing basic science questions in neuroscience and answer them with increasing sophistication. We can now study and specify the genetic, molecular, cellular, and circuit architecture of a brain region in much greater detail, which allows us to piece together how they contribute to emergent circuit functions. For instance, adopting such systematic and unbiased bottom-up approaches to elucidating the function of the visual system has paved the way to building a greater understanding of the spectrum of its computational capabilities. In the same vein, we argue that OFC research would benefit from a more balanced approach that also places focus on novel bottom-up investigations into OFC's computational capabilities. Furthermore, we believe that the knowledge gained by employing a more bottom-up approach to investigating OFC function will ultimately allow us to look at OFC's dysfunction in disease through a more nuanced biological lens.

Keywords: orbital frontal cortex, techniques, cell type, connectivity, macro and microcircuit function

Introduction

The origins of a field shape the direction of research for years to come. In the case of the orbital frontal cortex (OFC), seminal research was conducted in the second half of the 20th century which involved lesion experiments and behavioral observations made in non-human primates (Butter, 1969; Iversen & Mishkin, 1970). Several decades of ensuing experiments, recently focused on value-based decision-making, set the OFC field on a path towards pinning down what functional contributions this neural structure and its subdivisions make to cognitive processes (Stalnaker et al., 2015). As a result, the OFC field has been on a quest to identify the neural correlates of somewhat subjectively defined terms including choice, confidence, reward value, hedonic value, and decision.

Indeed, in trying to understand OFC function, the field often tries to relate the area's activity to specific aspects of behaviors that purportedly probe a theory of OFC function. This strategy has been valuable, especially with the use of operationally defined tasks designed to provide insight into the neural underpinnings of a particular psychological or cognitive construct (Sadacca et al., 2018; Shuck et al., 2016; Stalnaker et al., 2015; Wilson et al., 2014; Zhou et al., 2019). However, these findings should not be interpreted as the neural basis of the entire construct under scrutiny. Constructs derived from human reports and observations of animal behavior may not map so neatly onto any given neural circuit component or function. Litmus testing OFC function based on such constructs also ignores variations in experimental design that are likely to recruit different processes

(Balleine, 2019; Schreiner et al., 2020). Importantly, litmus testing does not further our understanding of the full spectrum of what the OFC can do. To broaden our knowledge, we also need to explore outside of the theoretical frameworks that have historically been emphasized in the field. While it is natural that the history of a field has a strong influence on its progress, scientific advancement, including in the realm of techniques and tools, should prompt periodic re-evaluation of what may have been overlooked or incompletely addressed along the way. We believe that the OFC field would benefit immensely from leveraging a rigorous, cutting-edge bottom-up approach to understanding the molecular, cellular, and circuit architecture of OFC and its control over OFC function and computational capabilities. For the purposes of this opinion piece, a bottom-up approach refers to building an understanding of OFC functions, starting from gene expression, cell-types, cell-type connectivity, all the way up to circuit-level readouts of neural activity in the context of behavior **(Figure 1.1)**. Conversely, a top-down approach is what has dominated OFC research, whereby theories about the gestalt function of the OFC get put to the test experimentally. To achieve a more balanced approach to furthering the field, we advocate for doing more of the former in the OFC, in part by taking advantage of the exciting new *in vitro* and *in vivo* techniques at our disposal.

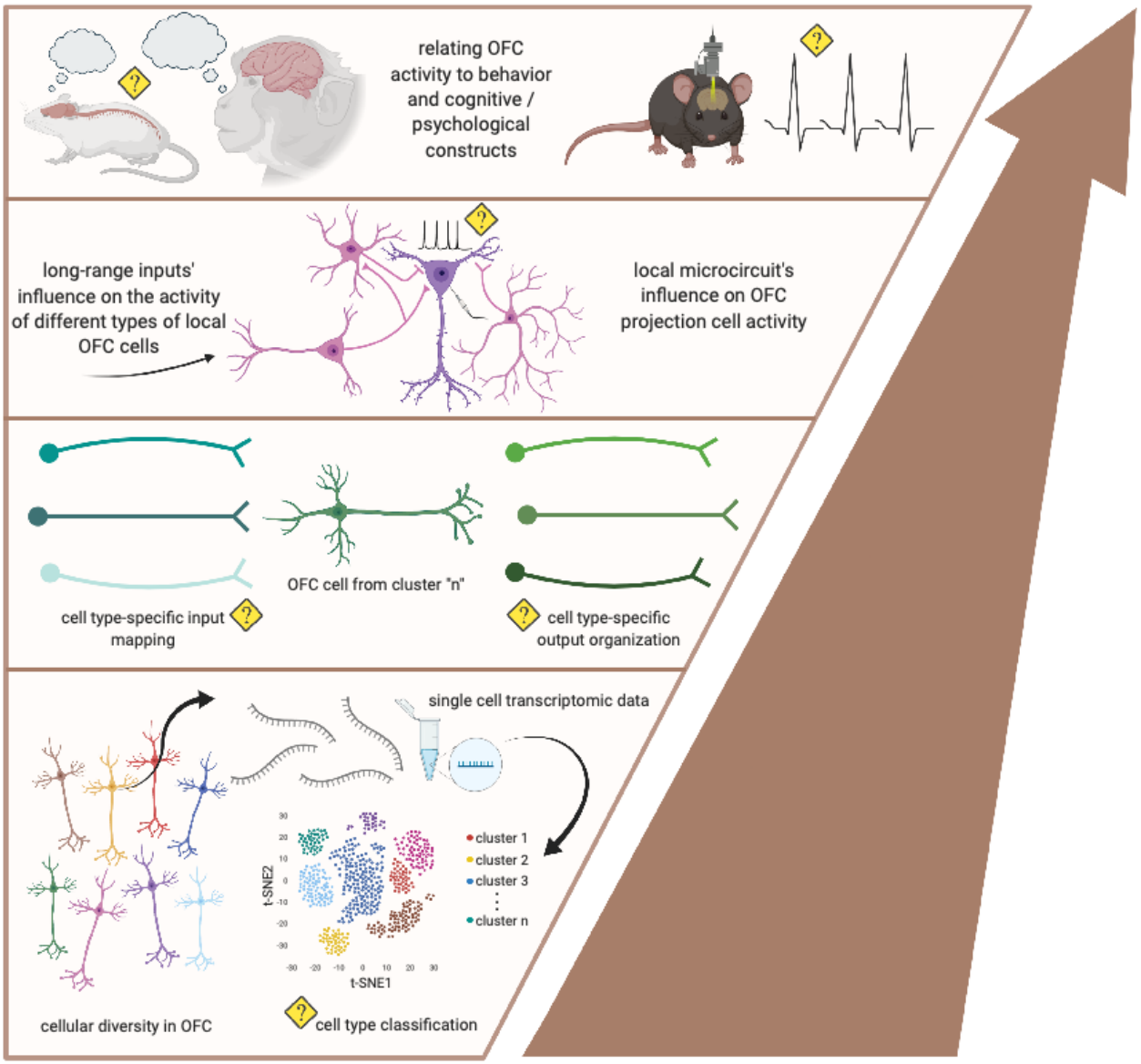

Figure 1.1: Schematic of a bottom-up approach to understanding OFC function. In the OFC field, there has been a lot of emphasis on relating OFC activity to particular aspects of behavior and human-defined cognitive or psychological constructs (top tier). In order to fully understand what OFC's unique functional contribution(s) to neural circuit computation and cognitive processes are, it would be beneficial to balance the relative dearth of experiments that study OFC function at the genetic, molecular, cellular, and circuit levels (bottom tiers). We argue for conducting more experiments that address the questions highlighted in the lower tiers of the schematic. Figure created with BioRender.

Another point we discuss is the fact that the field has largely centered on the activity of OFC itself while the finer details of how this neural structure is receiving, processing, and communicating information to other regions remain largely unexplored. In functional terms, what is OFC's place in this broader circuit? We ask this central question, akin to how scientists have considered the function of the primary visual cortex (V1) in the context of the visual information flow, starting from the retina all the way up to higher-order visual areas. Indeed, many neuroscientists contend that the most well-understood part of the brain today is the visual system. We hope the future direction of OFC research will achieve this level of understanding, as the need to understand OFC function is pressing given its reported disruption across numerous psychiatric disorders and diseases such as addiction, obsessive compulsive disorder, and depression (Drevets, 2007; Fettes et al., 2017; Goldstein & Volkow, 2011; Lüscher et al., 2020; Milad & Rauch, 2012; Pauls et al., 2014; Robbins et al., 2019; Schoenbaum & Shaham, 2008), as well as the growing potential for therapeutic approaches targeting the OFC (Fettes et al., 2017; Howard et al., 2020; Zilverstand et al., 2016). To that end, here we take the opportunity to map out a wishlist of experiments which we hope will help guide the field to gain more insight into OFC's computational capabilities and their disruption in disease.

1. A Bottom-Up Approach to Investigating the Circuit Components at Play in the OFC

We return to the visual system to highlight why we think it is crucial to take a deeper dive into the components of a biological circuit and how they connect when trying to glean circuit function. Since the 1950's, the accumulation of physiological findings in the context

of the known anatomy and connectivity of visual circuitry has led to an increasing understanding of how different cell types in the visual system contribute to the analysis of visual information. For instance, the structural study of the vertebrate retina was elaborated upon as techniques for staining and intracellular recordings were refined and put to use to catalogue the response properties of anatomically and morphologically identifiable cell types in the retina (Werblin & Dowling, 1969). Thus, scientists were able to systematically relate the responses of photoreceptor, horizontal, amacrine, bipolar, and retinal ganglion cells to varying patterns of illumination as well as to the response characteristics of their synaptic partners. By doing so, they were able to construct a working understanding of how the visual system begins to extract and relay features of the visual environment. Consequently, even though from a top-down perceptual perspective vision is a complex and multifaceted experience that involves contrast, form, depth, movement, color information, and more, scientists have been able to chip away at the fundamental questions probing the biological purpose and implementation of visual circuitry by working their way up from lower-level observations such as center-surround receptive field organization of certain cells at early stages of visual processing. The vision field has undoubtedly benefited from obtaining in-depth knowledge of the cellular diversity of the circuit as well as its input-output connectivity. This type of information is a starting point to deciphering what kind of information travels along the visual pathway and how that information is transformed and integrated at each level of processing. We believe that revisiting this type of circuit interrogation in the context of the OFC with new and more fine-grained tools will be extremely beneficial to understanding its capabilities.

A Meticulous Exploration of Cellular Diversity within OFC

Uncovering the vast cellular diversity of an evolutionarily more ancient brain region, let alone a prefrontal cortical region like the OFC, is staggering. With the emergence of cutting-edge single cell RNA sequencing (scRNA-seq) in conjunction with large-scale analyses on sequence-based data, we can now utilize transcriptomic data to characterize the diversity of OFC cell types across species as researchers have done in the mouse retina, V1, and other brain regions (Lein et al. 2017; Shekhar et al., 2016; Tasic et al., 2016; Wallace et al., 2020; Yuste et al. 2020). This would be a worthy large-scale endeavor, not only from a basic science and circuit dissection perspective, but also because disrupted OFC function is thought to contribute to numerous psychiatric disorders and diseases with genetic underpinnings that may affect different transcriptionally identified cell types in disparate ways (Fettes et al., 2017; Hernandez et al., 2020).

By combining scRNA-seq for marker gene identification with multiplexed error-robust fluorescence in situ hybridization (MERFISH) for imaging these marker genes in situ, it is now possible to identify and image hundreds of different RNA simultaneously with high accuracy in the cells' native spatial context (Moffitt et al., 2018). With the combined power of these two types of data, not only can OFC cells be classified based on transcriptomic similarity using unsupervised clustering analysis methods, but the spatial organization of the clusters can be observed in OFC as well. These molecular tools can shed light on the proportions of transcriptomically-defined cell types within previously identified subdivisions of OFC, as well as the spatial distribution of these cell types within and across OFC subdivisions. Furthermore, scRNA-seq, MERFISH, and immediate-early-gene expression imaging can be used together to identify and locate

clusters selectively activated by specific behaviors (Moffitt et al., 2018). Cataloguing these types of data can be useful for informing structure-function hypotheses that have persisted about OFC subregions. Indeed, we should leverage this approach to examine whether previously identified anatomical and functional subdivisions hold true from a transcriptomic classification perspective, or whether the divisions need to be revisited and/or expanded upon. Unknowingly treating a group of cells or a subdivision as anatomically or functionally homogeneous could hamper progress in our understanding of OFC. As an example of how these new approaches can mitigate such assumptions, through employing a combination of scRNA-seq and MERFISH in the preoptic region of the hypothalamus, researchers realized that galanin-enriched neurons, which had been hitherto treated as a unitary cell population, in fact segregated into multiple transcriptionally, spatially, and importantly, functionally distinct clusters (Moffitt et al., 2018).

Once OFC clusters are identified in an unbiased fashion, we can look for differential expression of gene families such as those for neurotransmitter and neuromodulator receptors, ion channels, and neuropeptides in order to glean insight into the cellular properties of each cluster (Paul et al., 2017). An added benefit to detailing the cellular diversity within OFC using this transcriptomic approach is the potential to identify robust cell type-specific markers for precise genetic targeting of various types of OFC neurons. This would pave the way for a higher level of cell type specificity when performing manipulation (e.g. optogenetic or chemogenetic) and imaging experiments in OFC (e.g. using calcium or voltage indicators). Thus, this is one avenue through which

the OFC field can gain a more nuanced understanding of the cell types at play in health and disease.

Critically, this type of cell type classification should be done across species to arrive at convergent approaches for future research directions. Additionally, as with all techniques and tools, it is important to corroborate findings with complementary approaches and exert care when interpreting results, thereby avoiding falling prey to lumping cells into a category without rigorous methods of qualifying or quantifying why this should be the case. For instance, single-cell transcriptomics alone was found to be insufficient in comprehensively profiling cortico-cortical projecting V1 neurons (CCPNs). Superficial layer CCPNs that fell into the same cluster based solely on transcriptomic data could be separated into statistically different clusters with systematic variation in gene expression if projection target and other connectivity information was taken into account (Kim et al., 2020). Therefore, efforts must be made in unity, combining molecular and state-of-the-art circuit tracing techniques, to define OFC cell types and their inputs and outputs. The results of such investigations would serve as an informational foundation and guide for circuit dissection and interrogation for the OFC field.

A Comprehensive Understanding of OFC Connectivity

Recent work on OFC connectivity has been guided by hypotheses about circuit function. For example, reciprocal connections between the lateral OFC and the basolateral amygdala (BLA) are thought to carry reward-related associative information critical for adaptive decision-making (Lichtenberg et al., 2017; Malvaez et al., 2019; Schoenbaum et al., 1998; Stalnaker et al., 2007). OFC's output through basal ganglia circuits may point to a role in contributing to action control (Hirokawa et al., 2019;

Kringlebach, 2004; Renteria et al., 2018), while its connectivity with midbrain monoaminergic neurons may suggest a role in reward learning and prediction (Chandler et al., 2013; Takahashi et al., 2011; Williams & Goldman-Rakic, 1998). Hypothesized functional roles of OFC inputs and outputs as well as decades worth of cytoarchitectural studies converge on the idea that OFC is made up of anatomically and functionally distinct subdivisions as mentioned in the previous section (Hoover & Vertes, 2011; Rudebeck & Rich, 2018). In rodents, OFC is generally understood to be composed of three subdivisions (medial, central, and lateral) with each further divided. Some have received greater attention than others due to a growing interest in their contributions to learning and value-based decision-making. For instance, rodent studies have suggested dissociable functional roles for medial versus lateral OFC projections to BLA in incentive learning; namely, reward value retrieval and value encoding/updating, respectively (Malvaez et al., 2019). Generally, in terms of anatomical and functional medial to lateral divide, a homology appears to exist between the human, primate, and rodent OFC (Heilbronner et al., 2016; Rudebeck & Murray, 2011; Wallis, 2011).

Yet, these anatomical definitions exist at a time when OFC heterogeneity is still not fully understood both within and across species. These definitions rely on limited OFC input-output mappings, and primarily center on excitatory projection neurons despite the rich array of GABAergic as well as glutamatergic neurons among cortical cell populations (Paul et al., 2017; Zeisel et al., 2015). Given the complexity of the circuit mechanisms that are likely to support OFC function, an unbiased approach to investigating connectivity of multiple cell types, including inhibitory neurons, will improve our understanding of OFC circuits.

i. Mapping OFC Input Connectivity

Identifying convergence of inputs can lead to a greater understanding of the target region and its broader circuit function. A striking example comes from the dorsal part of the visual thalamus, namely the dorsal lateral geniculate nucleus (dLGN). Researchers discovered that in addition to driver input from direction-selective retinal ganglion cells, the dLGN shell also receives driving synaptic input from visual motion selective neurons in the superior colliculus (Bickford et al., 2015). Through the identification of this anatomical convergence of excitatory inputs onto thalamocortical neurons in the dLGN shell and the functional validation of these synapses, they were able to posit how the direction-selective properties of dLGN neurons emerge from these structurally and functionally distinct parallel pathways. Figuring out that self-generated eye movement information from the superior colliculus and externally generated movement information from the retina converge at the level of the dLGN is a big step towards fully elucidating how the visual system tracks the trajectory of visual stimuli relative to eye movement. Thus, as this example illustrates, rigorous methods of identifying inputs can lead to not only anatomical, but important functional insights as well. Without this piece of the puzzle, it will be harder to conceptualize how OFC is able or unable to accomplish its functions in health or disease.

Monosynaptic rabies tracing is an exploratory technique that allows for brain-wide identification of direct inputs onto specific cell types (Callaway & Luo, 2015). While anterograde and retrograde tracers have been used to visualize inputs onto sizable portions of OFC (Murphy & Deutch, 2018; Roberts et al., 2007), the rabies tracing method confers the advantage of labeling presynaptic neurons that project to a highly specific set

of starter cells in an unbiased fashion. Starter cell type specificity can be achieved by making use of driver lines, most commonly Cre lines. The method uses a glycoprotein deleted rabies virus (RVdG) pseudotyped with the avian sarcoma leukosis virus glycoprotein EnvA (EnvA - RVdG) to selectively infect Cre-expressing starter neurons. The infection, complementation, and retrograde spread of EnvA - RVdG is enabled by Cre-dependent adeno-associated helper viruses (AAV) expressing the EnvA receptor (TVA) and the rabies glycoprotein (Luo et al., 2018). The retrograde spread is monosynaptically restricted because the input neurons lack the glycoprotein required for further spread of the virus. Thus, monosynaptic rabies tracing can readily label direct inputs onto genetically identified OFC neurons without any need for a priori knowledge of input identity.

Crucially, this viral tool makes it possible to investigate overlap or separation in input distributions onto genetically identified glutamatergic, GABAergic, and other types of local OFC neurons. Additionally, through combining anatomical and transcriptional profiling of an input region with rabies tracing from genetically specified OFC neurons, we can start to formulate more informed and fine-grained hypotheses about how OFC is being influenced by the input it receives. Using this strategy, a recent study identified distinct lateral habenular neuronal subtypes that differentially input onto dopaminergic and GABAergic neurons in the ventral tegmental area (Wallace et al., 2020). Indeed, precise and extensive data on input distributions onto OFC cell types will provide a much needed foundation to understand and test potential circuit effects of afferent inputs onto OFC. For example, feedforward inhibition through long-range excitatory afferents synapsing onto both local GABAergic interneuron populations and the glutamatergic

principal cell population is thought to be one potential mechanism by which timing of principal cells' activity is coordinated (Isaacson & Scanziani, 2011; Kepecs & Fishell, 2014). This circuit interplay, among other hypotheses on the variety of cortical GABAergic interneuron function (e.g. gain control through feedback inhibition, gating information flow in relation to specific events, orchestration of network oscillations), is likely extremely relevant for OFC function. Yet, without knowing which long-range inputs converge or diverge onto which of the various cell types in OFC, it is difficult to understand why the principal excitatory cell population in OFC behaves the way it does in response to a given input(s).

Rabies tracing can also be useful for studying OFC inputs in the context of pathology. For instance, changes in proportion of rabies-labeled inputs can be observed following a salient experience like drug exposure. Using this technical approach, a recent study showed how administration of drugs of abuse increases globus pallidus externus inputs onto genetically identified ventral tegmental area subpopulations critical for experience-dependent behaviors (Beier et al., 2017). Parallel work revealed that chronic cocaine administration leads to a significant increase in the proportion of cocaine-activated OFC neurons synapsing onto cocaine-activated dorsal striatum neurons following acute cocaine exposure, highlighting the potential power of this method for investigating circuit alterations in disease (Wall et al., 2019). Monosynaptic rabies tracing can also be utilized to perform functional manipulations of specific inputs onto a cell population of interest by having the rabies virus carry an excitatory or inhibitory opsin gene (e.g. ChR2) (Tian et al., 2016). Of note, rabies tracing can be limited by factors such as variable expression of glycoprotein in starter cells, and the eventual death of neurons

infected with the rabies virus (~14 days). However, working around these limitations and capitalizing on this tool's advantages will lead to a more comprehensive understanding of OFC input connectivity in health and disease, starting with the major OFC cell types identified as described in the previous section on cellular diversity.

ii. Moving Beyond Known OFC Output Organization

OFC has also been studied in the context of its projection targets. As mentioned previously, OFC has several cytoarchitecturally defined subregions of interest with a variety of projections stemming from them, including projections to dorsal and ventral striatum, as well as several direct reciprocal connections with brain regions ranging from the mediodorsal thalamus, amygdala, and parahippocampal gyrus (Fettes et al., 2017; Hoover & Vertes, 2011; Kondo & Witter, 2014; Schilman et al., 2008). However, we do not yet have a comprehensive understanding of what sets these different OFC cortical projection populations apart from a transcriptomics perspective, nor do we know if there is a higher-order organizational architecture to OFC projection populations that would supersede projection target identity. While categorizing projection neurons of a given region by their synaptic targets is a good starting point in the absence of more detailed information, goal-oriented usage of new molecular and genetic tools has demonstrated that we can take a large-scale and agnostic approach when investigating the output organization of a brain region.

For example, recent work in the thalamus discovered a novel framework through which projection neurons can be categorized based on molecular profiling (Phillips et al., 2019). Following retrograde labeling of thalamic projection neurons by viral or tracer injections in the projection fields of thalamic nuclei and RNA-sequencing of fluorescently

labeled thalamic cells, a hierarchical clustering analysis was performed on the five hundred most differentially expressed genes. As a result, three main gene expression profiles of thalamic projection neurons emerged, which interestingly did not cluster based on projection target. In fact, each injected projection field received input from all three genetically defined profiles. Notably, they discovered that the three profiles have a topographical arrangement along the mediolateral axis of the thalamus, and through access to previously published post-mortem human thalamus microarray data, they determined that this organizational feature is conserved in humans. In addition, they found prominent differences in electrophysiological properties (e.g. ion channel and receptor gene expression, action potential width) and axo-dendritic morphologies (e.g. axonal targeting of layers) across the three major transcriptional profiles of thalamic projection neurons. These findings are inspiring, because they demonstrate how large-scale bottom-up insights gained into the molecular and related topographical architecture of a brain region's outputs can help formulate hypotheses about functional variations housed in the region. Such systematic investigation of OFC output populations could also elucidate how these projections are poised to functionally influence the activity of their targets. Given that transcriptional differences underlying output populations can reflect differences in functional capabilities, conducting such an exploratory investigation could also aid in our understanding of how identified OFC output populations may differentially contribute to disease states.

Examining the Local Microcircuitry in OFC

To address what OFC can do and precisely how it can accomplish various circuit operations, we have already highlighted that we need to conduct more cutting-edge basic

science investigations at the molecular, cellular, and connectivity level. Now turning to the functional circuit-level, we argue that tapping into the full spectrum of OFC's capabilities may require conducting more detailed *ex vivo* and *in vivo* electrophysiology experiments examining local OFC microcircuitry, with the goal of elucidating how OFC activity arises and is modulated. Indeed, largely missing from the field is a more nuanced understanding of how the activity of a given OFC neuron and groups of OFC neurons are shaped by local activity dynamics to contribute to the representation, transformation, or communication of information.

A starting point can be to ask: What are the inputs and mechanisms driving an OFC neuron to fire (or not)? One simplistic, though extremely unlikely, possibility is that an OFC pyramidal neuron's activity is a read out of incoming excitatory inputs. More likely is a dynamic interaction between long-range inputs and the local cell types that dictates sub- and suprathreshold activity of OFC pyramidal neurons. This is a difficult puzzle to solve without knowledge of OFC's local circuit components, hence why we have argued for rigorous and comprehensive profiling of cell types and their spatial distributions in OFC. Luckily, through work in other cortical regions, researchers have begun to identify and understand how individual circuit components such as interneurons contribute to the computational capabilities of cortical microcircuits (Adesnik, 2017; Blackwell & Geffen, 2017; Ferguson & Cardin, 2020; Kato et al., 2017; Petersen, 2019; Yu et al., 2019).

Perhaps some of the more conclusive and striking observations made regarding local circuit influence on cortical pyramidal neuron activity come from sensory neuroscience. Again, if we look to vision research, surround suppression (i.e. the relative reduction in neural firing when a visual stimulus is enlarged such that the neuron's

receptive field surround is stimulated) has been a known phenomenon for decades. However, it was not until relatively recently that researchers were able to investigate the cortical circuit component that mediates this phenomenon at the level of V1, thereby disproving the idea that surround suppression is a feature entirely inherited from earlier stages of visual processing. By genetically accessing and manipulating specific cortical interneuron types while extracellularly recording the activity of V1 pyramidal neurons *in vivo*, researchers were able to ascertain that somatostatin-positive (SOM+) GABAergic cells are the local interneuron type driving the cortical component of surround suppression (Adesnik et al., 2012; Nienborg et al., 2013). Since technological advancements have made whole-cell recordings in awake head-restrained animals possible (Petersen, 2017), SOM+ cells' causal role in surround suppression has been confirmed in V1 on a single-cell level (Adesnik, 2017).

A related circuit operation that is an emergent function of local SOM+ cell activity is lateral inhibition. In sensory cortices, lateral inhibition typically refers to the ability of an excited pyramidal neuron to indirectly -- through its connectivity with SOM+ cells -- reduce activity of its neighboring pyramidal neurons with differing receptive fields. Recent work in the auditory cortex (A1) has demonstrated that fluctuating arousal state modulates SOM+ cell-mediated lateral inhibition and consequently the frequency tuning of A1 pyramidal neurons (Lin et al., 2019). Other work in the prefrontal cortex has examined how serotonin alters the intrinsic electrophysiological properties of parvalbumin-positive (PV+) GABAergic interneurons which are thought to be important for synchronizing oscillations. Their results suggest that serotonin increases PV+ cells' ability to temporally summate inputs at the high gamma frequency (Athilingam et al., 2017). These findings

point to the added complexity we are faced with when trying to understand the microcircuit dynamics of a prefrontal cortical region, like OFC, that receives neuromodulatory inputs in addition to a whole host of other inputs (Walker et al., 2009). Even so, the aforementioned examples illustrate that the payoff of investing time into studying the functional capabilities of cortical microcircuits is high, with the added potential of identifying principles of cortical dynamics that can aid our understanding of what goes awry in pathologies (Ferguson & Gao, 2018; Liu et al., 2020; Scheggia et al., 2020).

Increased accessibility of genetically identified cell types and the resulting ability to manipulate their activity in a temporally precise manner using optogenetics have benefited *ex vivo* investigations into microcircuits in addition to *in vivo*. The controlled environment of slice electrophysiology experiments allows one to examine the relative strength of incoming inputs, the balance of converging excitatory and inhibitory inputs, the likelihood of neurotransmitter release, mechanisms regulating presynaptic release, and the response properties of the various postsynaptic neurons. All of these mechanisms can modulate the activity profile of OFC pyramidal neurons. We will highlight examples of such mechanisms in the later section on OFC and disease, but want to emphasize here that more detailed *ex vivo* physiological experiments targeting specific OFC inputs and specific types of local cells will provide much needed information about how an OFC pyramidal neuron's activity is governed.

2. Investigating OFC Activity During and Outside of Task-Related Behaviors

Through the field's top-down perspective on OFC's role in cognitive processes, we have gained tremendous insight into the information OFC neurons are capable of

representing. Historically, this has been done using *in vivo* extracellular electrophysiology to examine OFC activity during discrete task epochs, though recently, calcium imaging experiments have also contributed insight. Recent work probing economic value (Cai & Padoa-Schioppa, 2019; Kuwabara et al., 2020; Yamada et al., 2018), inferred value (Baltz et al., 2018; Jones et al., 2012; Zhou et al., 2019), subjective decision (Cromwell, 2018; Rich & Wallis, 2016), sensory information (Saddaca et al., 2018), task structure (Zhou et al., 2019), and associative memory (Namboodiri et al., 2019) have continued to shed light on what OFC is capable of encoding. In addition, research targeting the functional contribution of specific OFC projection populations is expanding (Gremel et al., 2016; Groman et al., 2019; Liu et al. 2020; Malvaez et al., 2019; Namboodiri et al., 2019; Pascoli et al., 2019; Renteria et al. 2018; Schreiner & Gremel, 2018), and there has also been growing interest in investigating the activity of subpopulations within OFC, either based on their purported genetic identification (Bissonette et al., 2015), and/or their response profiles in relation to certain behaviors or task features (Jennings et al., 2019; Namboodiri et al., 2019).

The vast majority of work ascribing function to OFC has focused on using defined behavioral tasks or instructed movements (e.g. lever press, nose poke, or other means of reward pursuit) to investigate the area's contributions to cognitive processes. In this pursuit, analyses of neural activity have mostly focused on "task-related" neurons. Pertinent questions to ask are whether OFC's functional contributions are limited to discrete epochs of a defined task (Rich & Wallis, 2016) and whether exclusively focusing on neurons with large firing rate modulations during such epochs is sufficient to understand OFC's computational capabilities (Wallis, 2018).

Historically, *in vivo* extracellular recordings from OFC have been subject to trial-averaged analyses of unit activity. This has been the case even though trial-averaged firing rates of neurons do not always overtly relate to behavior or task-relevant features (Wallis, 2018). Indeed, these analyses typically hone in on units that demonstrate significant up- or down-modulation during discrete epochs of a task. However, a unit deemed "unresponsive" in a particular context, task set up, or temporal window is not necessarily functionally unimportant for the aspect(s) of the behavior or cognitive process at hand. Rather, it may be that we were unable to capture that unit's functional contribution to the process. As seen in the visual cortex, this issue can arise due to not sampling a large or diverse enough stimulus space (Garg et al., 2019), and becomes an even bigger, perhaps unsolvable problem in higher-order cortical areas where neurons are likely tuned to an even greater number of dimensions. All in all, by excluding "unresponsive" units from analyses, not only are we potentially discounting functionally relevant cells, but we may also be ignoring an important component of information that is embedded in a neural feature other than firing rate, such as spike timing (Insanally et al., 2019). Thus, instead of relying on our preconceived top-down understanding of what constructs are represented in OFC and how they're represented, we could benefit from less constrained behavioral and analytical approaches that leave room for additional hypotheses which could be experimentally verified.

With ongoing technological developments that allow large-scale recording of cortical activity and tracking of behavior, task-related and non-task-related neural activity as well as their relationship to behavior can be investigated more broadly. A striking observation that was made recently is that visual cortical population activity contains an unexpectedly

high dimensional structure, both during spontaneous activity and when mice are passively viewing visual stimuli (Stringer et al., 2019). A surprisingly sizable fraction of population activity variance could in fact be accounted for by a high dimensional measure of the mice's ongoing facial motor movements. Given that the V1 population encodes multidimensional behavioral information in addition to sensory information when mice passively view visual stimuli, one wonders how integration of information occurs at higher levels of cortex under more complex task scenarios. Complementary work using wide-field calcium imaging on the dorsal cortex of mice performing a decision-making task confirmed that, even in the context of a defined task, a variety of uninstructed movements such as whisking or hindlimb flexions accounted for more trial-by-trial variability in neural activity than instructed task-related movements or variables (Musall et al., 2019). This persisted in well-trained animals, and held true for thousands of neurons in various brain regions as well as across three different methods of activity measurement (i.e. high-density extracellular recordings with Neuropixels, two-photon imaging, and widefield imaging).

Given this observation that varied movements dominate single-trial cortical activity dynamics during a decision-making task, and that they can even be encoded at the level of V1 along with sensory information (Stringer et al., 2019), it can be speculated that this phenomenon underlies the prevalence of seeing similar apparent task-related activity in multiple different brain regions. This is critical to consider and account for, because in our goal of trying to understand what OFC is uniquely contributing to cognitive processes, we would do well to adopt some of the methods for extracting the task-related component of neural activity from movement related activity (if indeed separate, as movement can also

be a conditioned behavior) and other confounds (Musall et al., 2019). For a prefrontal cortical region like OFC, a big question is how to deconstruct such mixed representations in a way that allows us to identify what the region uniquely contributes to neural circuit computation. Examining OFC activity in instructed tasks that take into account additional factors such as movement, passage of time, and experienced history, would also provide a richer framework to investigate OFC contributions. Future work may benefit from studying OFC in the context of more ethologically-relevant paradigms (akin to viewing a natural scene in the case of studying the visual system).

Excitingly, the function of OFC activity outside of instructed behavior has begun to be explored more. For example, recently, the activity of OFC neurons that selectively respond to task-free feeding or social interactions with juveniles was recorded using two-photon calcium imaging and manipulated at a single-cell level during behavior (Jennings et al., 2019). Although the behaviors were observed in a head-fixed set-up, and the constraints that head-fixation and other forms of restraint impose on behavior and its associated neural activity should not be minimized, these studies set the stage for future investigations into OFC function during a broader class of behaviors. The continued development of techniques and tools with smaller footprints, such as the next generation miniaturized head-mounted fluorescence microscopes, will further such investigations by enabling large-scale imaging of the activity of genetically identified OFC populations during many different kinds of behavior, including relatively free behavior (Aharoni & Hoogland, 2019; De Groot et al., 2020; Flusberg et al., 2008).

3. Elucidating OFC Function Through the Study of OFC-related *In Vivo* Phenotypes in Disease

The prominence of varied *in vivo* disease phenotypes and their relation to altered OFC function illustrate the necessity of building a better understanding of OFC function. Here we want to re-emphasize that, as OFC is part of complex circuits, we should avoid the trap of limiting OFC's altered function in disease to specific behaviors and frameworks. We do not mean to imply that continuation of research aimed at identifying OFC's contribution to particular behaviors such as value-based decision-making is not warranted; indeed, much progress has been made with these types of investigations. Instead OFC, as part of intricate circuits, is likely to be involved in multiple functions contributing to numerous circuit computations and related phenotypes. We would argue that one extremely useful avenue through which to probe OFC functional capabilities is investigating altered OFC function in disease states. Importantly, gaining such an understanding would help improve and add to OFC-targeted treatments for therapeutic benefit (Howard et al., 2020; Zilverstand et al., 2016).

Altered OFC function has been associated with many disease-related *in vivo* phenotypes that have been observed in individuals with addiction (Goldstein & Volkow, 2011; Lüscher et al., 2020; Schoenbaum & Shaham, 2008) and obsessive compulsive disorder (OCD) (Milad & Rauch, 2012; Pauls et al., 2014; Robbins et al., 2019), among many others. While in some cases these disrupted behavioral phenotypes have been associated with heavily researched areas like value-based decision-making, often they are not. A notable example is altered OFC function in OCD and other related phenotypes (Lüscher et al., 2020; Pauls et al., 2014; Robbins et al., 2019; Wood & Ahmari, 2015).

While the field has at times suggested that OFC does not play a role in action control (Balleine et al., 2011; Fellows, 2007; Rudebeck et al., 2008; Wallis, 2007) (but see Bradfield et al., 2015; Gremel & Costa, 2013; Parkes et al., 2017; Rhodes & Murray, 2013), findings from the OCD literature strongly suggest otherwise. For instance, compulsions in OCD are associated with hyperactivity of OFC-striatal circuits (Milad & Rauch, 2012; Pauls et al., 2014; Robbins et al., 2019). This has been supported by findings from animal studies: One study observed that potentiation of OFC input into striatum is associated with compulsive grooming behaviors (Ahmari et al., 2013), while another found that OFC lesions increase lever pressing in a signal-attenuated lever press task (Joel et al., 2005). Similarly, OFC-striatal input potentiation has recently been implicated in compulsive, addiction-like behaviors, with mice continuing to press a lever for intracranial self-stimulation of dopamine neurons despite the presence of a punishing footshock (Pascoli et al., 2018). Associated increases in OFC terminal activity were observed specifically during the lever press period. This corresponds with prior work that showed OFC neurons can represent action-related information (Gremel & Costa, 2013). Thus, the activity of OFC neurons and their output to at least striatum has been strongly associated with aspects of control over action performance. This hyperactivity associated with OCD stands in stark contrast to the hypoactivity and deficient value-based decision-making often associated with addiction (Lüscher et al., 2020; Schoenbaum et al., 2016; Volkow et al., 2007). That addiction and OCD share some similar behavioral phenotypes including compulsion and insensitivity to reductions in value (Everitt & Robbins, 2016; Lüscher et al., 2020), but are often associated with opposite activity profiles, highlights the need to understand how disease states alter specific OFC computational capabilities.

In this light, the continued arguments about whether or not OFC plays a role in action control seem nonproductive. Crucially, it is likely that additional OFC-related *in vivo* phenotypes will be uncovered through disease investigations. Thus, we would argue for the continued identification of OFC-related phenotypes in disease models because they provide a golden opportunity to investigate mechanisms regulating OFC's capabilities as well as aid in identifying novel therapeutics.

Indeed, much of the information that we do have about structural, molecular, and cellular plasticity mechanisms regulating OFC function have come from investigations performed in disease models. Research from the addiction field shows that drug dependence disrupts sensitivity to value-based decision-making (Everitt & Robbins, 2016; Gremel & Lovinger, 2016; Lüscher et al., 2020), and changes structural and intrinsic properties of OFC neurons, as well as transmission onto and from OFC neurons. For example, alcohol dependence across species has been associated with increases in spine density on (McGuier et al., 2015) and reduced excitability of OFC projection neurons (Nimitvilai et al., 2017; Renteria et al., 2018) (but see Nimitvilai et al., 2016). Further, rescuing this reduced excitability restored goal-directed control over value-based decision-making (Renteria et al., 2018). Enhanced glutamatergic signaling onto OFC projection neurons has also been reported in a rodent model of alcohol dependence (Nimitvilai et al., 2016), converging with findings from non-human primate models of chronic alcohol drinking that point to changes in glutamatergic signaling (Nimitvilai et al., 2017). Changes to OFC structure and function are certainly not limited to alcohol dependence, and have been seen with psychostimulant exposure as well (DePoy & Gourley, 2015).

Addiction is not the only disorder associated with changes to OFC transmission and plasticity. Induction of obesity has also been shown to alter OFC's capabilities, altering the structural morphology of its projection neurons and reducing inhibitory transmission onto OFC neurons (Thompson et al., 2017) in part through regionally-specific mu opioid receptor driven suppression of presynaptic GABA release (Lau et al., 2020). As mentioned before, studies in the addiction and OCD fields have identified disease/disorder-induced changes to downstream OFC transmission that may contribute to value and action control, with potentiation of OFC input onto striatum in OCD models (Pascoli et al., 2018; Pascoli et al., 2015). Meanwhile, in alcohol dependence, there is reduced transmission from OFC onto the direct but not indirect pathway in the striatum (Renteria et al., 2018). Taken together, a picture begins to emerge pointing to ways in which diseases and disorders may affect afferent drive onto OFC, local microcircuit gating over OFC activity patterns, and OFC efferent activity, thereby disrupting or enhancing a specific OFC contribution to a given behavior.

Overall, basic science investigations into disease provide an opportunity to a) probe OFC function when behavior has been altered and b) build a better understanding of OFC's capabilities. Current findings have likely just scratched the surface of potential mechanisms involved. Given the broad innervation of OFC by modulatory systems such as serotonin (Puig & Gulledge, 2011) and dopamine (Loughlin & Fallon, 1984), which have strong clinical relevance, investigations into even more nuanced and specific mechanisms gating OFC computations are likely to be highly productive. We would do well to base investigations into psychiatric disorder and disease on the informational foundation provided by the techniques and approaches discussed in preceding sections,

in doing so greatly furthering our understanding of altered OFC capabilities. Given the clinical implications these findings would have, the possibility of opening additional avenues for treatment exploration is in itself sufficient to warrant further bottom-up basic science investigations into mechanisms underlying OFC's functional capabilities.

Conclusion

Understanding the pieces of a puzzle and how they relate to one another can help lead to the whole. Currently, we lack detailed and comprehensive information regarding the classification, spatial organization, connectivity profiles, and physiological properties of OFC neurons that would aid in building more informed hypotheses regarding OFC function. Substantial progress towards understanding OFC's functional capabilities may depend on the accrual of such information. Here, we suggest the employment of the full range of modern neuroscience techniques at the genetic, molecular, cellular, and circuit levels to answer the question, what can OFC do? In addition to continuing to investigate OFC function within the realm of decision-making, we argue there is a pressing need to understand how OFC contributes to a larger variety of circuit computations and behavioral phenotypes. This is especially pertinent as OFC shows altered function across a wide array of psychiatric disorders and diseases. A more inclusive and balanced investigative approach will provide a broader understanding of OFC's functional capabilities, thereby opening the door to novel and/or more effective therapeutic treatment strategies.

Author Contributions

All authors contributed to the conception and writing of this manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

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CHAPTER TWO: Orbitofrontal cortex projecting mediodorsal thalamic population activity reflects instrumental outcome expectancy

Abstract

Goal-directed behavior often relies on an internal representation of task parameters which, if updated appropriately based on experienced outcomes, ideally minimizes performance errors and uncertainty in predictions. The orbitofrontal cortex (OFC) is thought to be a key player in goal-directed decision-making, and is often studied on its own even though it is embedded in broader cortico-striatal-thalamo-cortical loops. OFC's prominent thalamic synaptic partner is the mediodorsal thalamus (MD) whose projections into lateral OFC have been repeatedly identified anatomically, but functional insights into how it is dynamically contributing to OFC processing have remained scarce. In freely behaving mice, we recorded the activity modulation of MD terminals in lOFC during a self-initiated chain instrumental task using fiber photometry, and found that the activity of the population is reflective of reward expectancy that builds across repeated probabilistic seeking actions distal to reward. Furthermore, animals' latency to press the lever proximal to reward is modulated by expectation, and affected by optogenetically inhibiting the activity of lOFC projecting MD somas. These findings are of interest given that lOFC neurons have been thought to encode behavioral contingencies, expectation, the reward statistics of a task structure / environment among other decision variables, but what information OFC's prominent synaptic partners like the MD may be providing to the local OFC circuit to achieve more accurate outcome predictions has remained underexplored.

Introduction

It has been established that the orbitofrontal cortex (OFC) is involved in the control of instrumental actions and maintaining an up-to-date representation of their associated outcomes (Gremel & Costa, 2013, Baltz et al., 2018). More generally, there is a fair amount of consensus in the field that the OFC is important for adapting an internal model of how external and internal variables relate to one another such that in a given context, accurate predictions can be generated in the service of flexible goal-directed behavior (Gardner & Schoenbaum, 2021). However, how exactly OFC may be accomplishing this function, and which sources of inputs onto OFC may be providing critical information or functional support for optimal flexible behavior are relatively unknown.

The mediodorsal thalamus (MD) is topographically connected with the prefrontal cortex, one of its primary targets being posterior-lateral OFC (Xu et al., 2021). Outside of results from functional OFC-MD disconnection studies or lesions of MD proper, there has not been much known about how MD input into lateral OFC may be contributing to functions that are thought to rely on the OFC (Fresno et al., 2019, Corbit et al., 2003). In particular, there has not been much investigation into the endogenous activity patterns of this influential input into lOFC during the learning and performance of instrumental tasks. Here, we sought to examine and manipulate the activity of the MD terminal population local to the lOFC during a self-initiated chain instrumental task in order to better understand what aspects of a goal-directed task MD-lOFC inputs may be contributing to.

Materials and Methods

Animals

Mice were housed 2–5 per cage under a 14/10 hr light/dark cycle with access to food (Labdiet 5015) and water *ad libitum* unless stated otherwise. Mice were at least 5 weeks of age prior to intracranial injections and at least 7 weeks of age prior to behavioral training. All surgical and behavioral experiments were performed during the light portion of the cycle. The Animal Care and Use Committee of the University of California San Diego approved all experiments and experiments were conducted according to the NIH guidelines.

Instrumental Task

We adapted an incentive learning task previously used in rats (Balleine and Dickinson, 1998; Wassum et al., 2009) to mice (Baltz et al., 2018). Regular food and water were freely available prior to the start of training. Mice were trained under food restriction (~1.5-2 gr/mouse of food daily and unlimited access to water in home cage) in standard sound-attenuating operant chambers with two levers flanking a food magazine containing a fluid well with contact lickometers, and a house light on the opposite wall (Med-Associates). Mice had to acquire a chain schedule of lever presses to receive a sucrose reward in the fluid well (20–30 µL of 20% sucrose solution per reward delivery).

Magazine training

On the first day, mice learned to approach the food magazine (no levers present) on a random time (RT) schedule, with a sucrose outcome delivered on average every 60 seconds for 30 minutes.

Continuous reinforcement

The next 3 days, mice had access to the right lever and right lever presses were rewarded on a continuous reinforcement (CRF i.e. fixed ratio one) schedule. By the end of CRF training, mice could earn up to 30 sucrose deliveries or the session would end after 60 minutes had passed. Fiber photometry mice could earn up to 60 sucrose deliveries by the end of CRF training (or the session would end after 90 minutes had passed). The maximum reward per session was higher for fiber photometry mice in order to collect more behavioral trials for analysis later on in training. Additional CRF training days were administered as needed.

Schedule training

Following CRF schedule training on the right lever, training continued with the introduction of the left lever. The session began with left lever out and right lever retracted. A left lever press on a random ratio one (RR1) schedule produced access to the right lever. Pressing the right lever on a FR1 schedule in turn retracted the right lever and produced a sucrose reward. On following days of training, the left lever requirement was increased to RR2, then RR4, and finally to RR8. The right lever was maintained on an FR1 schedule throughout training. During RR training, mice could earn up to 30 sucrose deliveries or the session would end after 60 minutes had passed. Fiber photometry mice could earn up to 60 sucrose deliveries per session in order to collect more behavioral trials for analysis (or the session would end after 90 minutes had passed). Response rates from the last 2-3 consecutive days of training prior to testing served as the baseline response rate. Mice with a response rate of 0.25 left lever presses per minute or less were excluded.

Surgical procedures

Mice were anaesthetized with isoflurane (1–2%) and intracranial injections were performed via Hamilton syringe (Reno, NV). Syringes were left in place for 3-5 minutes after each injection to allow for diffusion, and all viruses were infused at a rate of 100nL/min.

Fiber Photometry Recordings

Surgeries

To target expression of GCaMP to MD-lOFC projecting neurons, male and female C57BL/6J mice received stereotaxically guided injections of rAAV5/Ef1a-Cre-WPRE (UNC Virus Vector Core, unlabeled Cre, ~300-400 nL) via Hamilton syringe into lOFC (coordinates from Bregma: A, +2.65 mm; M/L, 1.85 mm; V, 2.6 mm) and Cre-dependent axon-enriched GCaMP injections (AAV5-hSynapsin1-FLEX-axon-GCaMP6s, Addgene, ~300-400 nL) into MD (coordinates from bregma: A, -1.34 mm; M/L, 1 mm; V, 3.6 mm at a 12° angle from ordinate for optimal targeting of MDm/c). A subset of mice simply received injections of axon-enriched GCaMP (AAV5-hSynapsin1-axon-GCaMP6s-P2A-mRuby3, Addgene, ~250 nL) into MD. Multimode optical fibers (Thorlabs, 400 micron core, 0.39 NA) were implanted 0.2 mm above GCaMP-expressing MD-lOFC axon terminals in lOFC. For experiments involving imaging of putative lOFC excitatory population, just rAAV5/CamKIIa-GCaMP6s (UNC Virus Vector Core, ~300 nL) was injected into lOFC, and fiber-optic ferrules implanted at the aforementioned OFC coordinates (V, 2.4 mm). Mice were given at least 2-3 weeks to allow for recovery from surgery and viral expression before the start of experimental procedures. After

experiments were completed, mice were euthanized, and their brains were extracted and fixed in 4% paraformaldehyde. Viral expression, spread, and optical fiber placements were assessed in 100-150 µm-thick brain slices using a fluorescence microscope (Olympus MVX10, CellSens software).

Data Collection

During operant training, ceramic mating sleeves (Thorlabs, ADAL1) were used to couple the steel ferrule implants (Thorlabs, 1.25 mm diameter, 440 micron bore) to a bifurcated patch cable (Thorlabs, 400 micron core, 0.39 NA), thereby allowing for simultaneous imaging of two mice per photometry set-up. A 470 nm mounted LED (Thorlabs, M470L3) was used for excitation of MD axon terminals in lOFC through the patch cable, and the light pulse was controlled by a custom Arduino script. Emission was collected through the same patch cable, hence the appropriate excitation and emission filters and light paths were set up accordingly using reconfigurable dichroic mirrors. The dual-fiber core was focused through a 4X objective (Olympus) onto a CMOS camera (FLIR Systems). Regions of interests (i.e. the respective fiber cores) were selected for each mouse using open-source Bonsai software (Lopes et al., 2015). Fluorescence intensity (sampled at 20 Hz) and incoming Med-PC TTL pulses (Med Associates Inc.) for lever extensions, presses, head entries, outcome deliveries, and licks were acquired simultaneously, thresholded, and time stamped using Bonsai. We did not employ a reference isosbestic channel in the 405–410 nm wavelength range, as GCaMP6s can be excited within that range; however, we did run control mice that just had fluorescent

protein (eYFP) expressed in lieu of GCaMP to account for Ca+2-independent emissions (rAAV5/hSyn-eYFP, UNC Vector Core, ~300 nL).

Data Preprocessing

Data was imported into Matlab (Mathworks Inc., Natick, MA) and analyzed based on prior works using custom scripts (Gremel Lab Github). A median filter and zero-phase lowpass filter were used to remove random noise artifacts while avoiding signal distortion, and a highpass filter with a low cut-off frequency (0.001 Hz) was used to correct for photobleaching across a behavioral session (Photometry data preprocessing.ipynb, Akam, 2019). Thus, all components of the fluorescence signal changing on a slower timescale than ~16 minutes were removed, thereby removing the drift due to bleaching and any slow physiological variation. For each behavioral session, we estimated baseline fluorescence (F0) by calculating the running 10th percentile of the preprocessed fluorescence signal trace using a 15 second left-sided sliding window. We calculated the normalized change in fluorescence (dF/F0) by subtracting the F0 trace from the preprocessed fluorescence signal trace and dividing the result by the F0 trace. Only behavioral sessions in which the 97.5% of dF/F0 across the session (independent of any behavioral response) exceeded a 1% change were included (Markowitz et al., 2018).

Data Analysis

To align photometry and behavioral data, the Bonsai timestamps from the event-related TTL pulses were aligned to the closest Bonsai timestamps from the fluorescence sampling. For each session, ‘trials’ were composed of peri-event dF/F0 traces -5 to +10

seconds from event onset. In each 'trial', 50 ms bins were z-scored to the pre-event baseline dF/F0 (-10 to -5 seconds from event). We analyzed this photometry data using two approaches: In one approach, all the z-scored dF/F0 traces were combined across all mice and then averaged. This was done to preserve the variance seen within a subject. For the second approach, we averaged the z-scored dF/F0 traces from each mouse and then calculated the mean of these averages across mice. This approach examines between-mouse variability but does not preserve within-subject variability. We then were able to conduct statistical comparisons between peri-event z-scored traces obtained from experimental (GCaMP6s) and control (eYFP) mice as well as shuffled data traces. Bootstrapping was used to estimate confidence intervals for each time point and to identify temporally-defined significant differences from null (dF/F0=0) in the peri-event traces. For activity trace comparisons, we performed a running permutation test (10,000 random permutations, thus resolution of permutation p-values: $p < 0.0001$) for each time point in the peri-event traces (functions implemented in Matlab from Jean-Richard-dit-Bressel et al., 2020). To reduce Type I errors due to random fluctuations, the consecutive threshold to tag a transient as significantly different was 4 adjacent samples (i.e. 200 ms, double the low-pass frequency period). All such analyses were two-tailed with $\alpha = 0.05$ to determine significance. For peak (or trough) modulation, the mean of the four highest (or lowest) consecutive z-score values in a given 'trial' was calculated, and subsequently all of these mean values were averaged across trials. Data was analyzed and graphed using Matlab, Excel (Microsoft), and Prism (Graphpad).

Statistical Analyses

Behavioral data was analyzed using Excel (Microsoft), Matlab (Mathworks), and Prism (Graphpad). All analyses were two-tailed with $\alpha = 0.05$ as a threshold for significance. For analyzing coarse behavioral measurements (e.g. Lever Presses), one-way or two-way RM ANOVAs were used with Bonferroni corrections for post-hoc multiple comparisons unless otherwise noted. For cases in which comparison groups had significantly different standard deviations, Brown-Forsythe ANOVA test was used with Games-Howell's multiple comparisons tests. Latency behavior that did not appear to be distributed normally in histograms and QQ plots underwent tests of normality and lognormality, and log transformations were conducted before using parametric tests. Nonparametric Kruskal-Wallis tests with Dunn's multiple comparisons tests were used on other non-normal bounded data for which log transformations did not address the normalcy requirement.

Somatic inhibition of MD-lOFC neurons

In order to target our optogenetic manipulation to MD neurons projecting to lateral OFC (MD-lOFC neurons), we used a dual virus approach to express archaerhodopsin (ArchT) in a projection-specific manner (Han et al., 2011). Male and female C57BL/6J mice received stereotaxically guided bilateral injections of rAAV5/Ef1a-Cre-WPRE (UNC Vector Core, unlabeled Cre, ~300-400 nL) via Hamilton syringe into lOFC (coordinates from Bregma: A, +2.65 mm; M/L, 1.85 mm; V, 2.6 mm), as well as bilateral injections of rAAV5/FLEX-ArchT-tdTomato (UNC Vector Core, ~300-400 nL) into MD (coordinates from Bregma: A, -1.34 mm; M/L, 1 mm; V, 3.6 mm at a 12° angle from ordinate for optimal targeting of MDm/c). Control mice received injections of rAAV5/FLEX-tdTomato (UNC

Vector Core, ~300-400 nL) into MD instead of ArchT. Multimode optical fibers (Thorlabs, 200 micron core, 0.39 NA) were implanted at a 12° angle from ordinate and 0.2 mm above the MD viral injection sites to target light stimulation onto the somas of MD-lOFC neurons. Mice were given at least 2-3 weeks to allow for recovery from surgery and viral expression before the start of experimental procedures. After experiments were completed, mice were euthanized, and their brains were extracted and fixed in 4% paraformaldehyde. Viral expression, spread, and optical fiber placements were assessed in 100-150 µm-thick brain slices using a fluorescence microscope (Olympus MVX10, CellSens software).

Following recovery from intracranial injection and implant surgeries, mice were trained on the instrumental task. For optogenetic manipulation, from RR2 schedule training and onwards ferrule implants (Thorlabs, 1.25 mm diameter, 230 micron bore) were connected to fiber-coupled LEDs (Thorlabs, M595F2) in order to acclimate mice to handling and being tethered during behavioral sessions. Mice were lightly anaesthetized for attaching their ferrule implants to patch cables (Thorlabs, 200 micron core, 0.39 NA) via ceramic mating sleeves (Thorlabs, ADAL1), and were allowed 15-20 minutes of recovery before behavioral sessions started. First day of RR8 schedule training and onwards, ~50% of the time that mice met the schedule requirement on the left seeking lever, mice received 5 seconds of continuous 595 nm light stimulation (1–3 mW at optical fiber tip). For stimulating ArchT, using off-peak 595 nm light has been shown to minimize off-target light absorption effects while effectively suppressing neural activity across the desired volume of tissue (Setsuie et al., 2020). The high-powered 595 nm LEDs were controlled by custom Arduino scripts such that the start of light stimulation was triggered by a Med-PC TTL pulse (Med Associates Inc.). The efficacy of the inhibitory opsin ArchT

was validated *ex vivo* by patching onto tdTomato-positive MD-lOFC neurons and, while in current-clamp, optically stimulating with either 530, 590, or 625 nm light delivered onto the recording bath via field illumination (Thorlabs, LED4D067).

Brain slice preparation

Coronal slices (250 μm thick) containing the MD were prepared using a Pelco easiSlicer (Ted Pella Inc., Redding, CA). Mice were anesthetized by inhalation of isoflurane, and brains were rapidly removed and placed in 4°C oxygenated ACSF containing the following (in mM): 210 sucrose, 26.2 NaHCO3, 1 NaH2PO4, 2.5 KCl, 11 dextrose, bubbled with 95% O2/5% CO2. Slices were transferred to an ACSF solution for incubation containing the following (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 2.4 MgCl2, 1.8 CaCl2, 10 dextrose. Slices were continuously bubbled with 95% O2/5% CO2 at pH 7.4, 32°C, and were maintained in this solution for at least 60 min prior to recording.

Patch clamp electrophysiology

Whole-cell current clamp recordings were made in MD-lOFC neurons. MD cells that expressed ArchT were identified by the fluorescent tdTomato label using an Olympus BX51WI microscope mounted on a vibration isolation table and a high-power LED (Thorlabs, LED4D067). Recordings were made in ACSF containing (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 0.9 MgCl2, 2.0 CaCl2, and 10 dextrose, bubbled with 95% O2/5% CO2. ACSF was continuously perfused at a rate of 2.0 mL/min and maintained at a temperature of 32°C. Picrotoxin (50 μM) was included in the recording

ACSF to block GABAA receptor-mediated synaptic currents. Recording electrodes (WPI Instruments, thin-wall glass capillaries) were made using a PC-10 puller (Narishige International, Amityville, NY) to yield resistances between 3–6 MΩ. Electrodes were filled with (in mM): 135 KMeSO4, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Tris-GTP, 260–270 mOsm (pH 7.3). Access resistance was monitored throughout the experiments.

Current clamp recordings

Recordings were made using a MultiClamp 700B amplifier (Molecular Devices, Union City, CA), filtered at 2 kHz, digitized at 10 kHz with Instrutech ITC-18 (HEKA Instruments, Bellmore, NY), and displayed and saved using AxographX (Axograph, Sydney, Australia). A series of fixed current injections (20-30 pA increments from 0-300 pA) were used to elicit action potential firing. For verification of ArchT function, current injections were done with periods of 500 ms light OFF-ON-OFF. The number of elicited spikes when light was OFF vs. ON were counted and compared at each current step.

Results

MD terminal population in lOFC is differentially recruited during various epochs of a chain instrumental task

To address the question of how the MD-lOFC projection population may be contributing to OFC function during the learning and performance of a self-initiated goal-directed task, we first performed *in vivo* fiber photometry and measured population Ca2+ activity from axon-enriched GCaMP-expressing MD terminals in lOFC (n = 9 mice) **(Figure 2.1A-D)**.

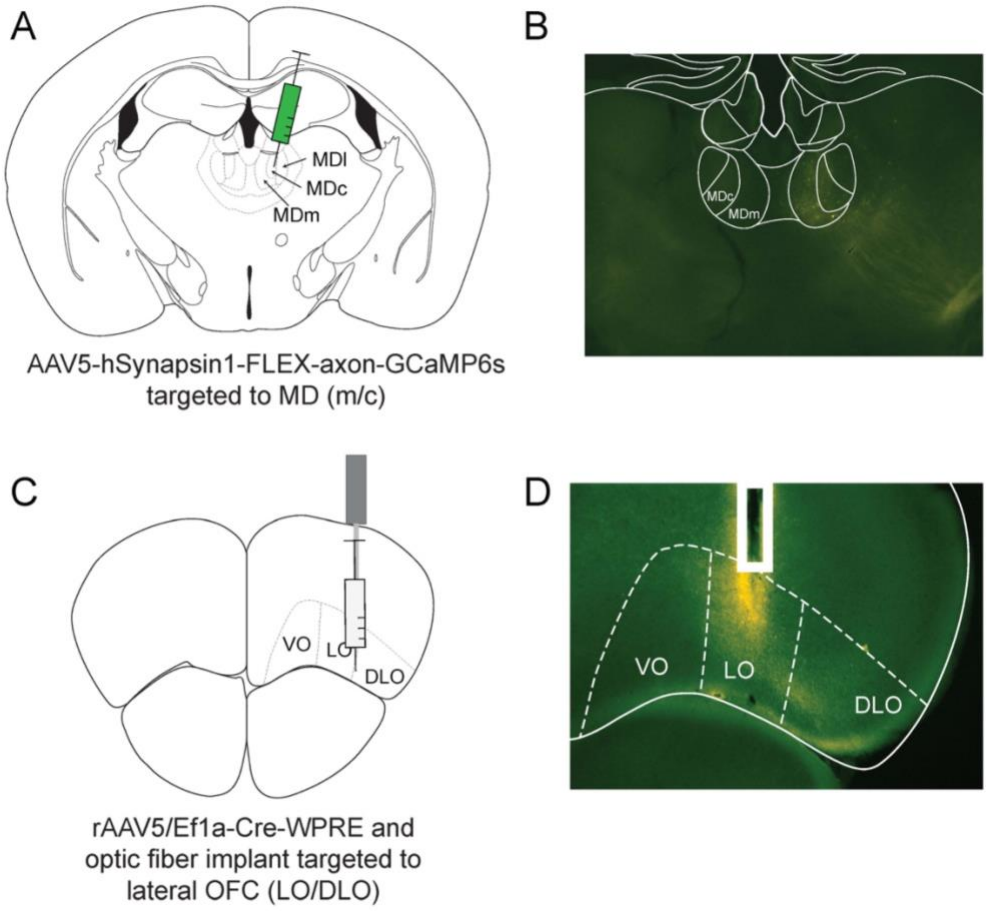

Figure 2.1: Dual-virus approach to achieve axon-enriched GCaMP expression in lOFC projecting MD neurons. (A) Schematic indicating the MD subregion that was targeted for unilateral axon-enriched GCaMP injection. **(C)** Schematic indicating the lateral OFC subregions targeted for unilateral Cre injection and optic fiber implant placement. **(B and D)** Cre was robustly picked up by MD thalamocortical terminals in lOFC to achieve Cre-dependent expression of axon-enriched GCaMP in central and medial MD. Axon tracks coming out of MD are visible in 2.1B. Bands of MD terminals in superficial and deeper layers of lOFC are visible in 2.1D.

We selected an instrumental task that we previously used to probe lOFC function (Baltz et al., 2018; Wassum et al., 2009; Corbit & Balleine, 2003) which required mice to learn to perform a chain lever press sequence for a reward (see Materials and Methods). In brief, a left 'seeking' lever which was always available needed to be pressed under a random ratio (RR) schedule in order to produce the right 'taking' lever. A subsequent right lever press under a fixed ratio 1 (FR1) schedule resulted in reward delivery **(Figure 2.2A-B)**. Across training, mice readily learned the contingencies and adapted their behavior to match the increasing RR schedule requirement on the left lever **(Figure 2.2C-D)**.

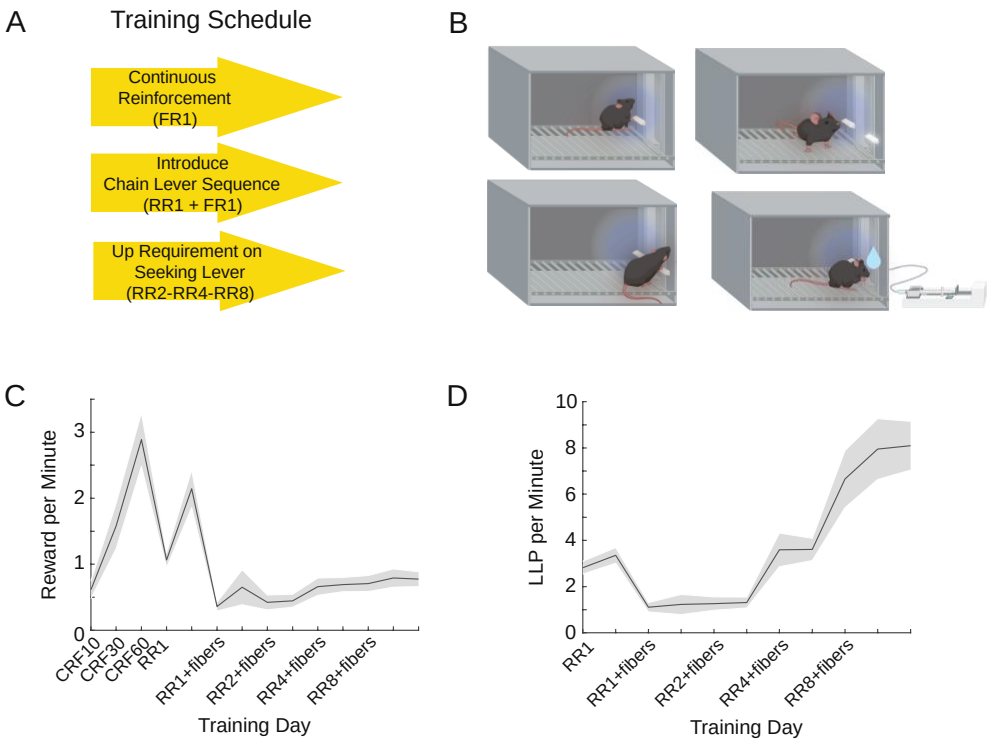

Figure 2.2: Chain instrumental task contingencies. (A) Order in which the contingencies were introduced. **(B)** Schematic of a mouse learning to press the left 'seeking' lever in order to produce the right 'taking' lever, which when pressed resulted in sucrose reward delivery. **(C)** Reward rate across training under a CRF (FR1) schedule, followed by the introduction of the chain rule (RR+FR1) and fiber coupling. Eventual stabilization of reward rate across RR training days as new contingencies were learned. **(D)** Left lever press rate across training as the RR requirement on the left seeking lever was increased. Data points are mean+SEM.

Of particular note, under an RR schedule, each action-outcome is independent of the previous iteration as each action has an equal probability of producing the outcome. By the end of task acquisition, an RR8 schedule dictated that each left lever press could produce the right lever with probability X=⅛. Thus, in this task context, the timing of the right lever coming out in a given trial was unpredictable, and functioned as a salient cue signaling the more proximal availability of potential reward.

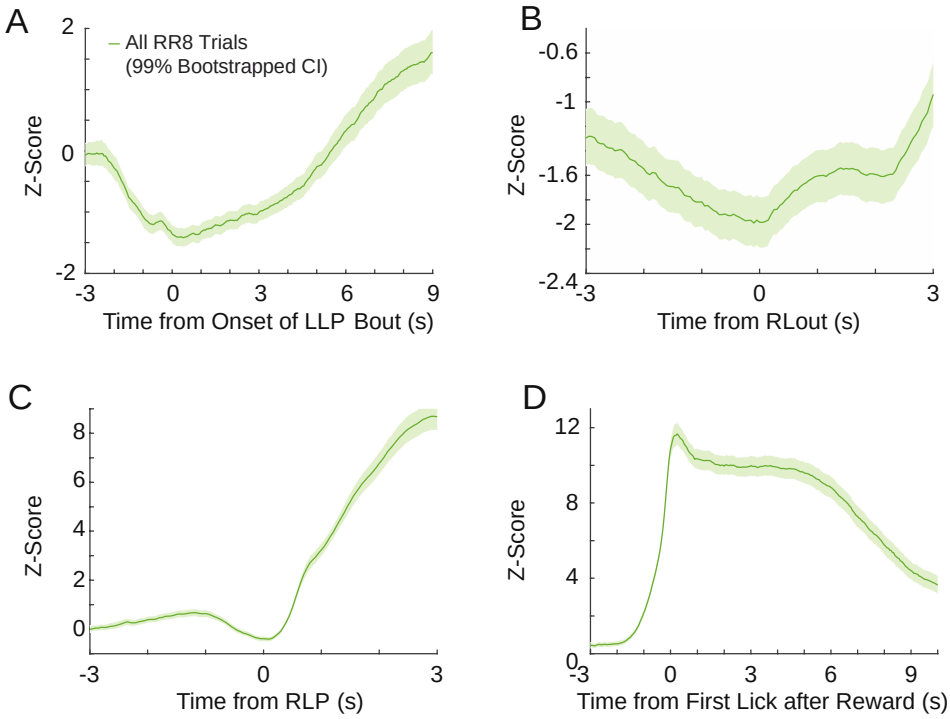

Figure 2.3: MD-lOFC terminal population activity modulation across task epochs. Ca^{+2} activity traces are averages with shaded 99% bootstrapped confidence intervals. Data reflects all trials from all mice. MD-lOFC terminal population activity modulation relative to pre-event baseline for **(A)** left lever press bouts, **(B)** the production of the right lever, **(C)** and right lever press. Pre-event baseline for 2.3B was taken from the first lever press of the LLPs that led to RLout for each trial in order to eliminate confounds related to differential amounts of left lever presses needed to produce the right lever across trials **(see Figure 2.5C-I)**. **(D)** Terminal population activity surrounding the start of reward consumption and subsequent licking behavior **(also see Figure 2.4C)**.

To assess MD-lOFC terminal population engagement during various task epochs, we aligned calcium activity to left lever press bout onset (LLP bouts), right lever out

(RLout, i.e. the same time point as the last left lever press that produces the right lever in a given trial), right lever press (RLP), and the onset of licking behavior following reward delivery **(Figure 2.3A-D)**. The general pattern of MD-lOFC terminal population activity modulation surrounding these epochs can also be observed on a trial-by-trial basis in peri-event heatmaps **(Figure 2.4A-C; representative mouse)**: Prior to the onset of LLP bouts, terminal population fluorescence signal decreased relative to baseline fluorescence and remained so throughout the LLP bout, suggesting reduced MD-lOFC terminal engagement during seeking actions **(Figure 2.4A)**. The reduced engagement was present until the last LLP that produced the right lever (RLout time point; **see Figure 2.3B**). There was a modest relative increase in terminal population activity following RLout, which was followed by a much larger increase in activity after RLP **(Figure 2.4B)**. Following RLP, mice typically moved to the central port and began to consume the sucrose reward that was concomitantly delivered with RLP. The persistent relative increase in terminal population activity throughout a licking bout was striking **(Figure 2.4C)**. Thus, the MD-lOFC terminal population seemed to be particularly engaged by reward-proximal cues and actions, as well as reward consumption.

That we observed bidirectional changes in activity modulation across key behavioral epochs in which a *probabilistic* seeking action schedule leads to a deterministic taking action-outcome contingency raises the possibility that MD-OFC terminal population activity dynamics may be sensitive to expectation-related information and/or influenced by reward anticipation.

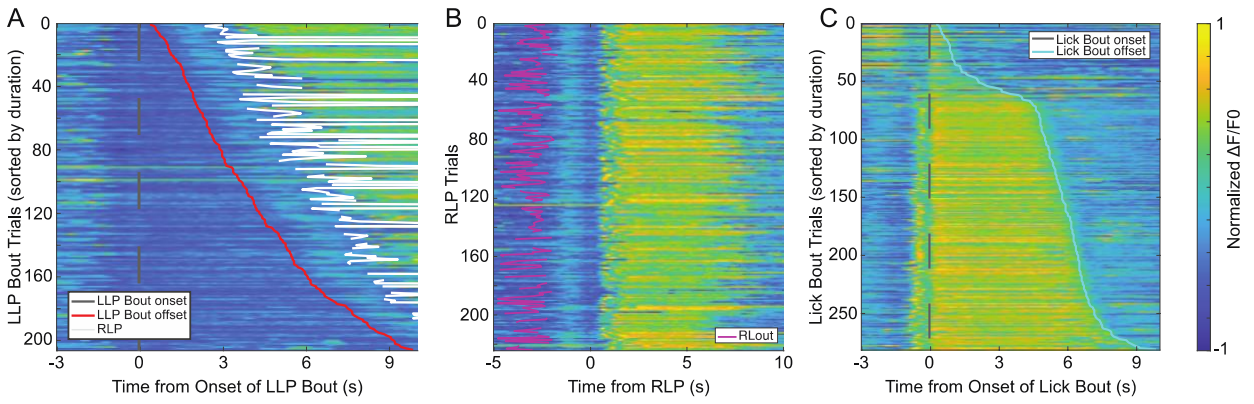

Figure 2.4: Trial-by-trial MD-lOFC terminal population activity from a sample mouse. Relative change in MD-lOFC terminal population fluorescence signal **(A)** throughout left lever press bout durations, **(B)** following right lever out and right lever press, and **(C)** throughout licking behavior.

MD terminal population activity in lOFC reflects expectation of reward-proximal cue and action

In order to quantify the spectrum of left lever pressing requirements mice experience under the random ratio schedule (RR8), we plotted the distribution of how many times mice had to press the left seeking lever to produce the right lever (**Figure 2.5A**; n = 12,694 trials). We divided the distribution into quartiles (Q1 = 1-3 left lever presses, Q2 = 4-6 left lever presses, Q3 = 7-11 left lever presses, and Q4 = 12+ left lever presses) and examined MD-lOFC terminal population calcium activity in relation to this quartile categorization. The distribution that each individual fiber photometry mouse experienced across its RR8 training paralleled the quartile boundaries derived from the overall distribution of LLP requirements from numerous training sessions (**Figure 2.5B**). The higher quartiles (Q3 and Q4) encompass trials during which mice had to press the seeking lever numerous times to produce the right lever – notable experiences, especially in contrast with their experiences under RR1, RR2, and RR4 schedules of training. Thus, we were motivated by the question of whether expectation built upon prior and ongoing

experiences in RR8 instrumental training would be reflected in the MD-lOFC terminal population activity categorized by quartile. Importantly, within this task structure, it is not possible to know *a priori* exactly which left lever press will result in right lever production in any given trial, as this contingency is under probabilistic control. Therefore, across trials categorized by quartile, we examined peri-event calcium activity leading up to the final left lever press which simultaneously results in RLout.

We found ramping down of MD-lOFC terminal population activity relative to a baseline period taken prior to the onset of each trial's left lever pressing (**Figure 2.5G-I**; 1-way ANOVA $F(3, 1453)=0.1932$, $p=0.9011$; Kruskal-Wallis 1.021, $p=0.7962$), and this pattern was present across quartiles (**Figure 2.6A**; inset **Figure 2.6B**).

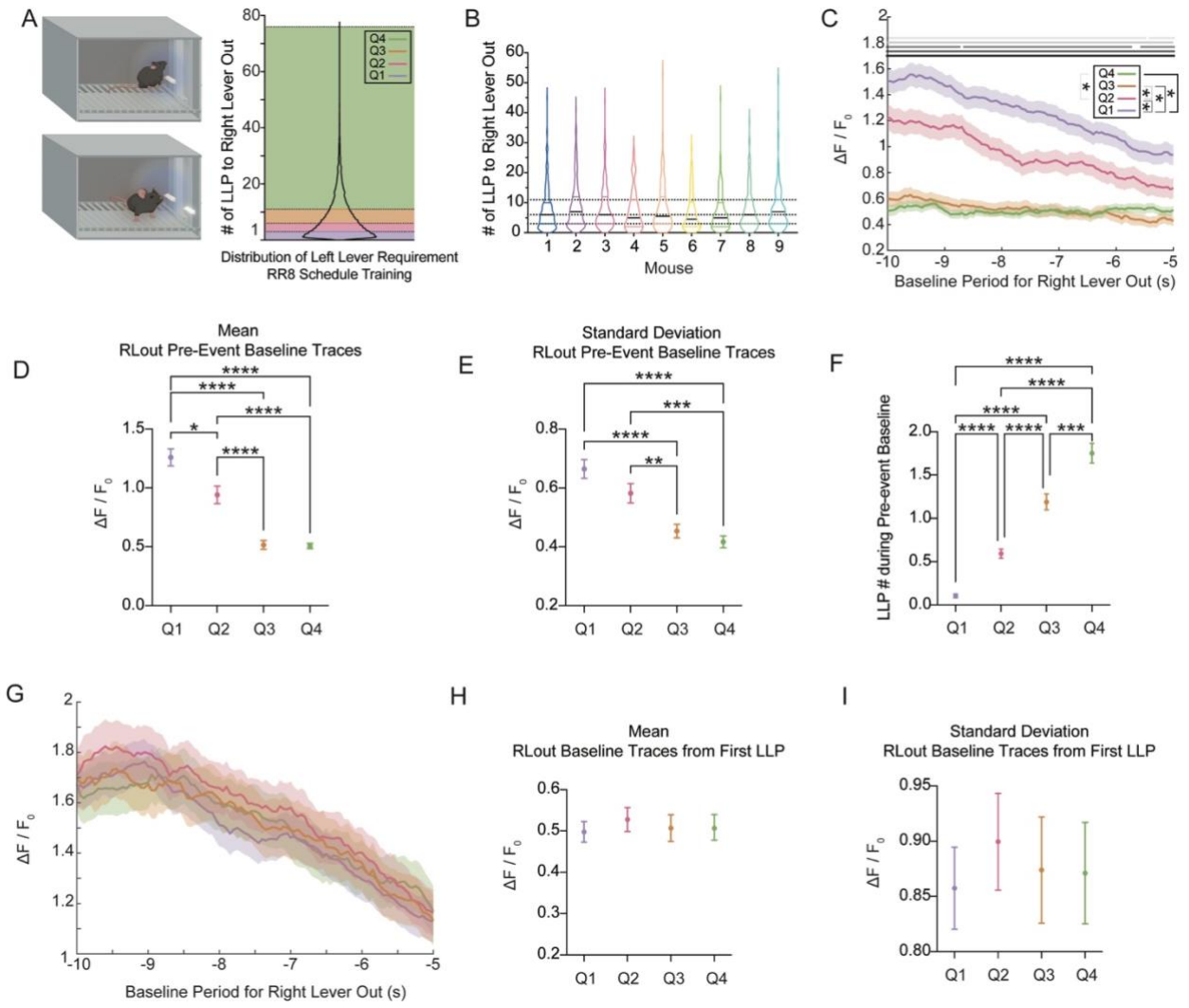

Figure 2.5: Distribution of seeking lever requirements and its categorization. (A) At the end of instrumental training, mice experience the chain lever press contingency under an RR8 schedule which yields different left lever press requirements across trials. (right) Violin plot (n=12,694 trials) of the distribution of LLP requirements that occurred under the RR8 schedule categorized into quartiles (Q1-Q4). **(B)** The distributions of LLP requirements that mice experienced during their respective RR8 training sessions. **(C)** Quartile differences in MD-lOFC terminal population Ca^{+2} activity during the pre-event baseline period for RLout as identified by permutation tests (10,000 random permutations, α = 0.05 for quartile comparisons). **(D)** Differences in means of pre-event RLout baseline traces across quartiles (Brown-Forsythe ANOVA F(3, 1018)=44.33, p<0.0001). (E) Differences in standard deviations of pre-event RLout baseline traces across quartiles (1-way ANOVA F(3, 1456)=12.29, p<0.0001). **(F)** Different amounts of LLPs that take place during the RLout pre-event window across quartiles (Kruskal-Wallis 292.4, p<0.0001). **(G-I)** Chosen baseline period for each RLout event is 5 seconds preceding the first LLP of that trial as this resolves the confound of differing pre-event RLout baseline activity across quartiles. **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05. Data points are mean+SEM.

Across quartiles, this decrease in MD-lOFC terminal population activity was followed by a relative increase in activity after RLout, as well as a subsequent bigger increase (data from sample mouse in **Figure 2.6D**). Permutation tests between quartiles revealed differences in MD-lOFC terminal population activity (see Materials and Methods): Specifically, prolonged statistically significant differences were observed between RLout peri-event activity from Q1 versus Q3 and Q4 trials (**Figure 2.6A**). At the RLout time point, Q4 trials had greater activity compared to Q1 trials (**Figure 2.6C**; 1-way ANOVA $F(3,1453)=3.041$, $p=0.0280$ and Bonferroni's multiple comparisons test, Q1 vs. Q4, adjusted $p=0.0396$), and this difference was present in these traces following RLout and after the start of reward consumption (**Figure 2.7A-D**; 1-way ANOVA $F(3, 1453)=3.815$, $p=0.0097$ and Bonferroni's multiple comparisons test, Q1 vs. Q4, adjusted $p=0.0105$; 1-way ANOVA $F(3, 1218)=1.553$, $p=0.1990$; 1-way ANOVA $F(3, 1094)=2.851$, $p=0.0364$ and Bonferroni's multiple comparisons test, Q1 vs. Q4, adjusted $p=0.0522$; 1-way ANOVA $F(3, 1094)=2.873$, $p=0.0353$ and Bonferroni's multiple comparisons test, Q1 vs. Q4, adjusted $p=0.0320$). The observed difference in MD-lOFC terminal population activity between quartiles could not readily be accounted for by the amount of left lever presses that occurred in the five seconds leading up to RLout (**Figure 2.6E**; Spearman correlation, $r = -0.1058$, $p<0.05$). The very weak negative monotonic correlation between RLout activity and number of LLPs performed in the 5 seconds leading up to RLout does not explain why there was greater RLout activity in Q4 trials compared to Q1 trials, because upper quartile trials contained more LLPs in that 5-second window compared to Q1 (**Figure 2.6F**; Kruskal-Wallis test 277.1, $p<0.0001$ and Dunn's multiple comparisons test, Q1 vs. Q4, Q1 vs. Q3, Q1 vs. Q2, adjusted $p<0.0001$).

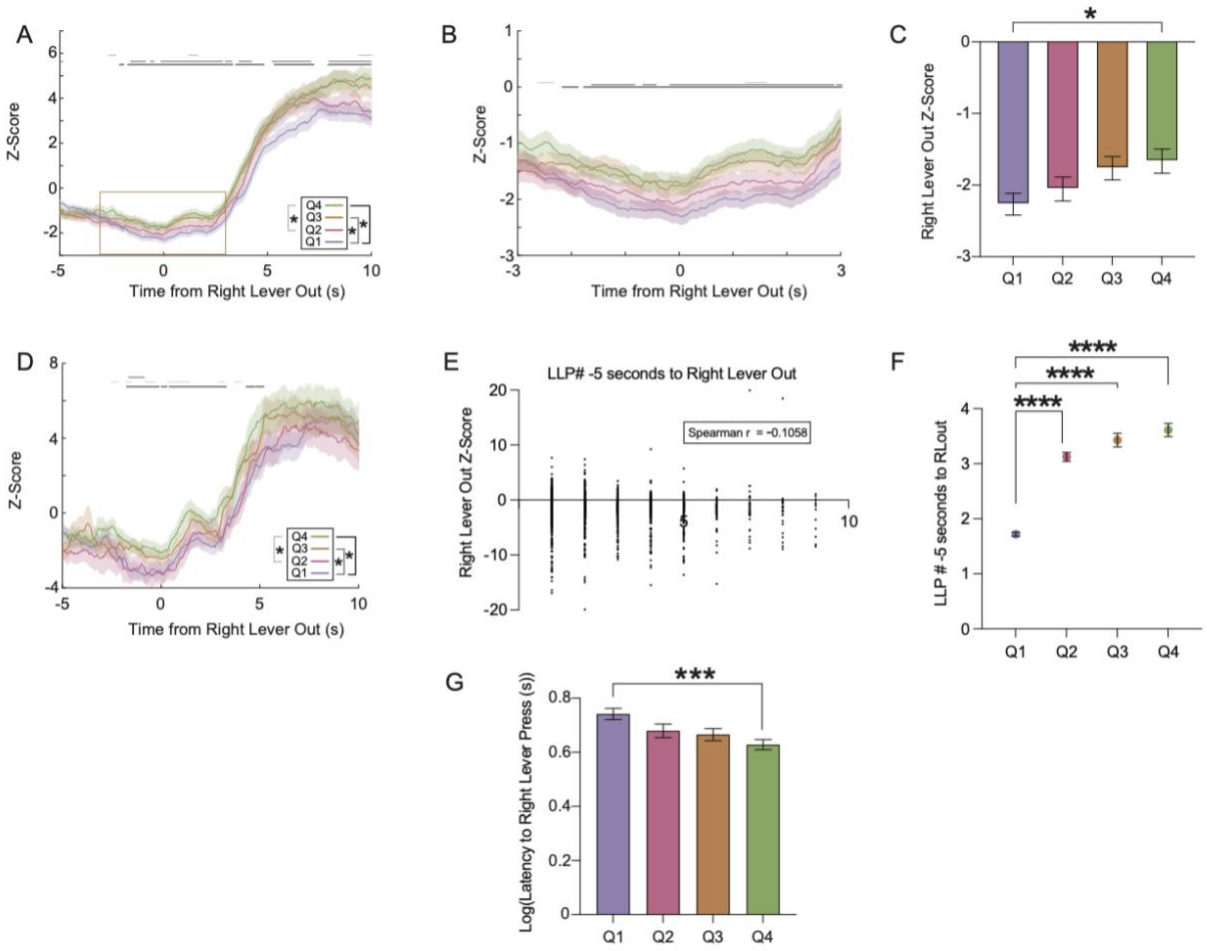

Figure 2.6: MD-lOFC terminal population activity modulation is sensitive to expectation that emerges as a function of executing an increasing number of reward-seeking actions. **(A)** Prolonged MD-lOFC terminal population Ca^{+2} activity quartile differences surrounding RLout as identified by permutation tests (10,000 random permutations, α = 0.05 for quartile comparisons). **(B)** Inset from 2.6A. **(C)** Activity differences at RLout across quartiles. **(D)** RLout peri-event MD-lOFC terminal population activity differences across quartiles in a sample mouse. **(E)** Very weak negative monotonic correlation between activity at RLout and amount of LLPs that occurred 5 seconds leading up to RLout ($p<0.0001$). **(F)** Quartile differences in number of LLPs performed in the 5 seconds leading up to RLout. **(G)** Differences in latency to RLP across quartiles. **** $p<0.0001$, *** $p<0.001$, * $p<0.05$. Data points are mean+SEM.

We subsequently examined MD-lOFC terminal population activity aligned to first lick after reward delivery across trials categorized by quartile. Permutation tests once again revealed prolonged statistically significant differences between Q1 versus Q3 and Q4 trials (**Figure 2.8A**) which was corroborated by peak modulation differences (**Figure**

2.8C; 1-way ANOVA $F(3,1447)=7.505$, $p<0.0001$ and Bonferroni's multiple comparisons test, Q1 vs. Q4, adjusted $p<0.0001$, Q1 vs. Q3, adjusted $p=0.0022$). These differences in MD-lOFC terminal population activity across quartiles could not be accounted for by meaningful differences in patterns of licking behavior (**Figure 2.8D-E**; 1-way ANOVA $F(3,1448)=2.653$, $p=0.0472$; 1-way ANOVA $F(3,1448)=0.5082$, $p=0.6766$). Taken together, these data suggest that MD-lOFC terminal population activity around reward-proximal and reward consumption task epochs is modulated by preceding expectation that is built as mice execute an increasing number of reward-seeking lever presses in a given trial.

We hypothesized that, in addition to neural activity, the animals' behavior could be modulated by expectation as a function of the increasing number of left lever presses performed under the RR8 schedule. We asked whether mice would exhibit different latencies to RLP depending on the trials' quartile categorization. We found that latency to RLP was indeed different across quartiles (**Figure 2.6G**; Brown-Forsythe ANOVA $F(3,1370)=5.417$, $p=0.0011$), with post hoc analyses showing significantly shorter latencies in Q4 trials when mice emitted the most number of left lever presses compared to Q1 trials when mice emitted the least number of lever presses (**Figure 2.6G**; Games-Howell's multiple comparisons test, Q1 vs. Q4, adjusted $p=0.0003$).

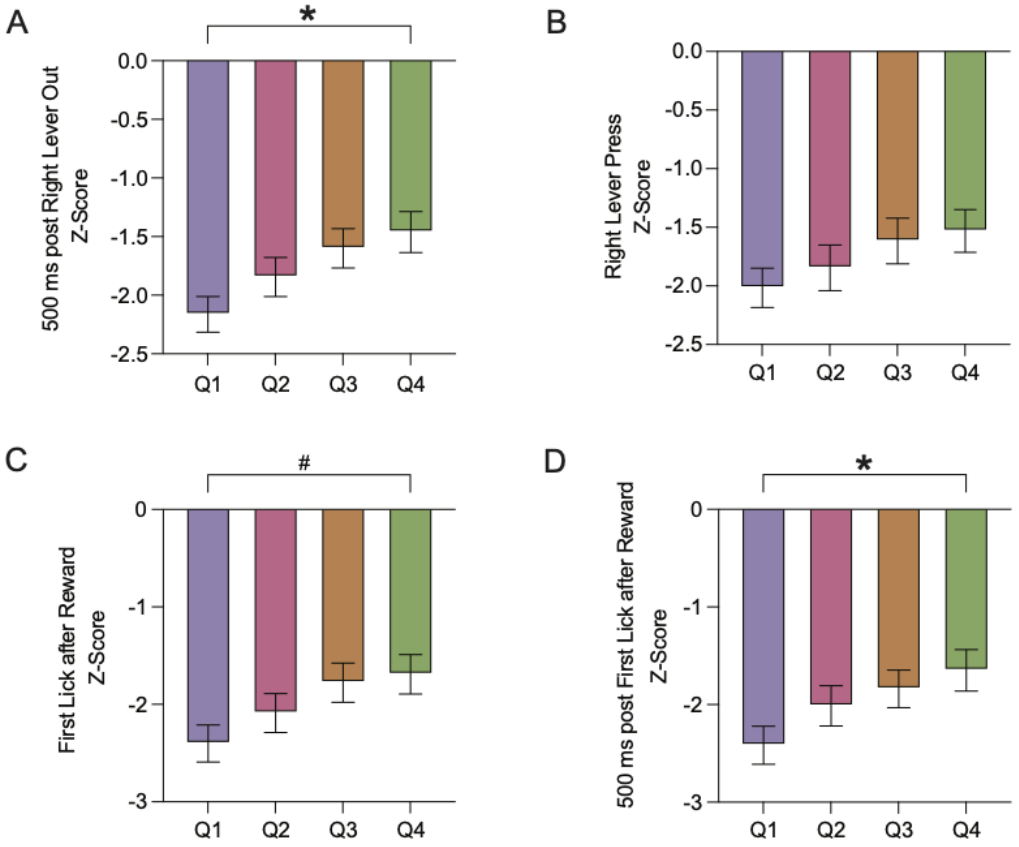

Figure 2.7: MD-lOFC terminal population activity differences between lowest and uppermost quartiles persist beyond right lever out time point. (A) MD-lOFC terminal population Ca^{+2} activity quartile differences shortly after the production of the right lever, **(B)** at right lever press, **(C)** at first lick after reward delivery, and **(D)** shortly after the first lick after reward delivery. Z-Score values at these timepoints were taken from peri-event activity traces aligned to RLout (**see Figure 2.6**). * p<0.05, #<0.06. Data points are mean+SEM.

In contrast, latency to first lick following RLP and concomitant reward delivery remained stereotyped across quartiles (**Figure 2.8F**; 1-way ANOVA F(3,1450)=1.712, p=0.1625). These observations suggest that in this task context, latency to RLP in particular can be influenced by expectation. This raises the question of whether the activity of MD-lOFC projecting neurons may be causally involved in modulating behavior that is sensitive to expectation.

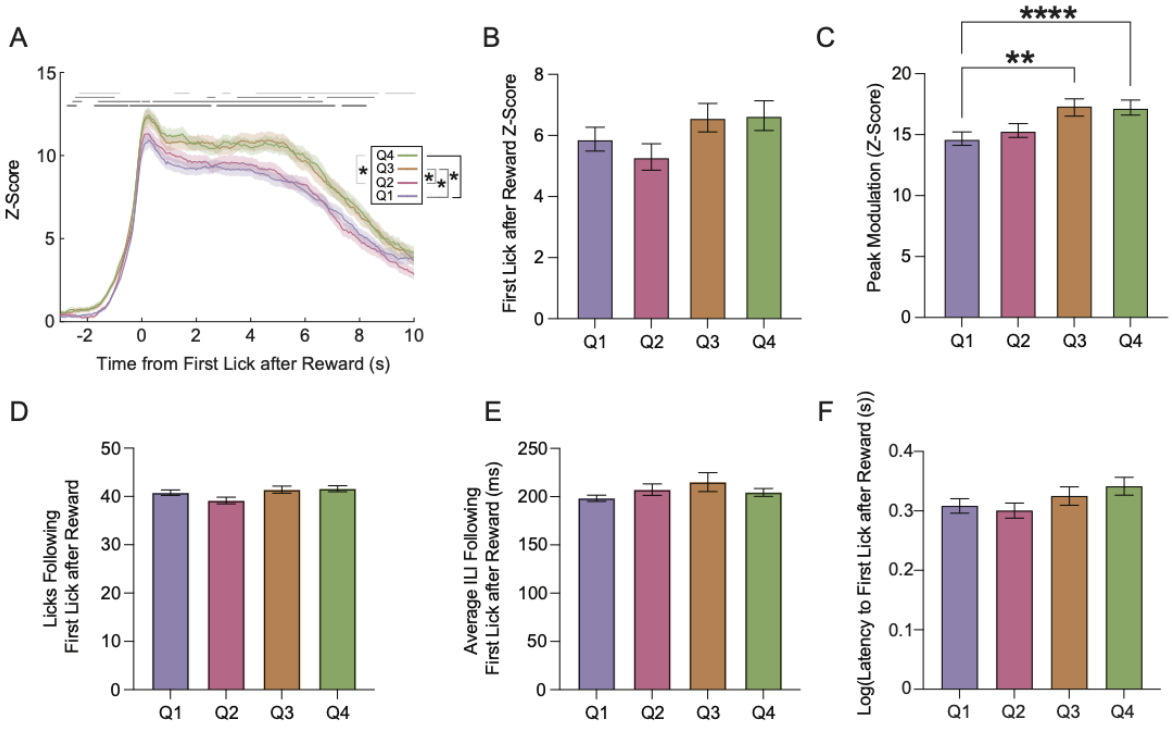

Figure 2.8: Reward consumption-related MD-lOFC terminal population activity differences across quartiles cannot be explained by licking behavior. (A) Prolonged MD-lOFC terminal population Ca^{+2} activity quartile differences surrounding first lick after reward delivery as identified by permutation tests (10,000 random permutations, $\alpha = 0.05$ for quartile comparisons). **(B)** Activity differences at first lick after reward delivery across quartiles. **(C)** Peak MD-lOFC terminal population activity modulation (i.e. average of 4 consecutive highest Z-Score values) within the time frame depicted in 2.8A across quartiles. **(D)** Number of licks following reward delivery within the 10-second data frame in 2.8A by quartile. **(E)** Average interlick interval following reward delivery within the 10-second data frame in 2.8A by quartile. **(F)** Differences in latency to first lick after reward delivery across quartiles. **** $p<0.0001$, ** $p<0.01$. Data points are mean+SEM.

Inhibiting MD-lOFC projection neurons renders mice suboptimal performers

We hypothesized that MD-lOFC terminal population activity provides information related to the expectation of reward underlying task performance. Thus, we asked whether manipulating the activity of MD-lOFC projecting neurons after completion of the RR8 schedule requirement on the left seeking lever (i.e. when the right lever is produced) would influence latency to RLP. We again used a dual-virus approach to express a variant of inhibitory opsin archaerhodopsin, ArchT, in lOFC projecting MD neurons (Han et al.,

2011) (**Figure 2.9A**). Cre was robustly picked up by MD thalamocortical terminals in lOFC to achieve Cre-dependent expression of ArchT in MD-lOFC neurons. We validated that this opsin functioned as expected in our soma population of interest *ex vivo* -- that is, upon continuous 590 nm light stimulation, we observed cessation of firing in cells expressing ArchT and receiving a current injection sufficient to elicit action potentials (**Figure 2.9B**). We triggered light activation upon the production of the right lever (**Figure 2.9C**), since RLout-related MD-lOFC terminal population activity and beyond differed across quartiles that engender differing reward expectancies (**see Figures 2.6-2.8**).

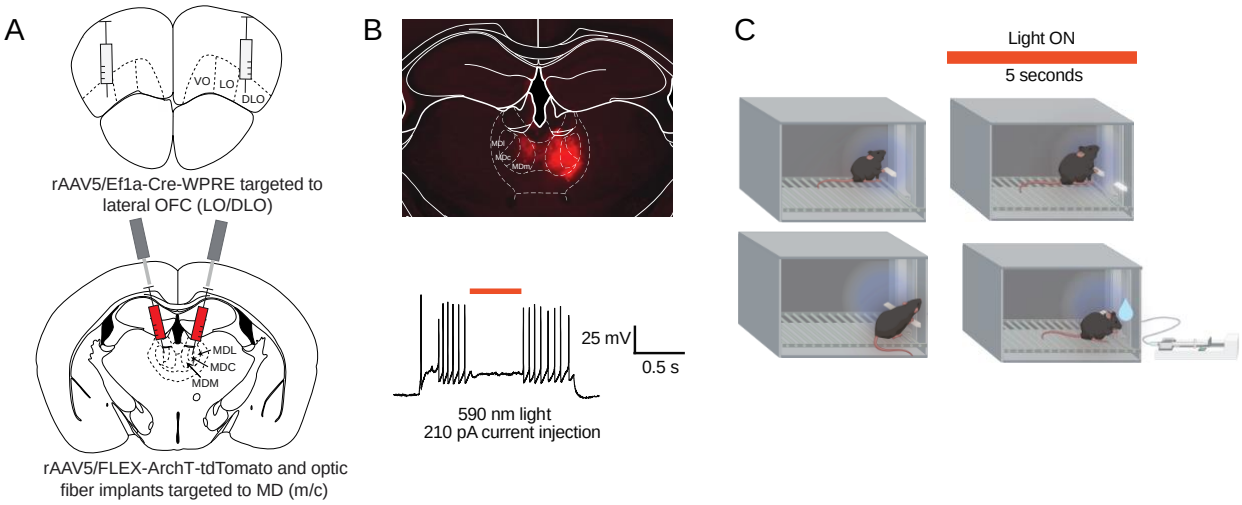

Figure 2.9: Dual-virus approach to expressing and validating inhibitory opsin ArchT in lOFC projecting MD neurons. (A) (top) Schematic of bilateral Cre injections into lOFC and (bottom) bilateral Cre-dependent ArchT injections into as well as optic fiber implants above central MD. **(B)** (top) ArchT expression in predominantely central-medial MD and (bottom) *ex vivo* validation that activating ArchT achieves inhibition of lOFC projecting MD cells. **(C)** In the context of the chain lever press task, light trigger was tied to the production of the right lever proximal to reward. Once triggered, light was on continuously for 5 seconds.

On a macro level, the experimental group did not differ in their instrumental learning across training from a control group expressing a red fluorescent protein instead of ArchT (**Figure 2.10A-C**; Mixed-effects model, no group effect or group by training day

interaction, F(1,13)=0.1740, p=0.6833 and F(11,121)=0.4475, p=0.9311; Mixed-effects model, no group effect or group by training day interaction, F(1,13) = 0.1188, p= 0.7359 and F(11,121)=0.5442, p=0.8695; Mixed-effects model, no group or group by training day interaction, F(1,13)=0.02261, p=0.8828 and F(11,121)=0.7142, p=0.7230). First day of RR8 schedule training and onwards, on random, ~50% of the time mice met the RR8 schedule requirement on the left lever they received 5 seconds of continuous 595 nm light stimulation.

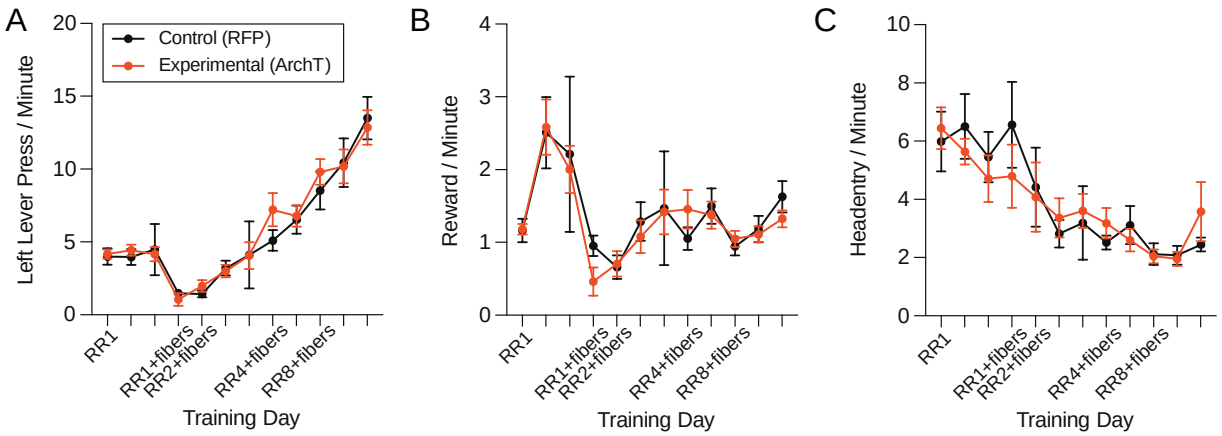

Figure 2.10: Experimental and control groups do not differ in their instrumental training at a macroscopic scale. (A) Increasing left seeking lever press rate with increasing RR requirement on the left lever across training. **(B)** Reward rate across training following the introduction of the chain rule. **(C)** Decreasing head entry rate across training following the introduction of the chain rule.

We calculated the latency to RLP across the two stimulation conditions and groups. We found that inhibiting MD-lOFC projecting somas upon RLout in a subset of trials resulted in global increased latency to RLP (**Figure 2.11A-B**; Kruskal-Wallis test 83.13, p<0.0001; 1-way ANOVA F(3,1912)=27.52, p<0.0001). This effect was present across both light on and off trials. One hypothesis could be that inhibiting MD-lOFC projecting soma activity interferes with expectancy-related signaling which can modulate responsiveness to the emergence of the right lever. To explore this possibility, we

examined whether mice were perseverating on the left seeking lever longer than they needed to in order to produce the right lever. We found that experimental mice persisted to a greater degree in their left lever pressing, beyond the necessary amount (ratio value > 1) (**Figure 2.11C**; Kruskal-Wallis test 52.97, $p<0.0001$). When we looked at mice's performance across stimulation days through the lens of optimal performance (i.e. pressing the left seeking lever only as much as needed to produce the right lever), we observed that inhibiting MD-lOFC projecting soma activity rendered mice suboptimal performers to a greater degree than control mice (**Figure 2.11D-F**; 2-way ANOVA, group $F(1,18)=2.770$, $p=0.1133$; 2-way ANOVA group, $F(1,18)=6.014$, $p=0.0246$; 2-way ANOVA, group $F(1,16)=3.754$, $p=0.0705$).

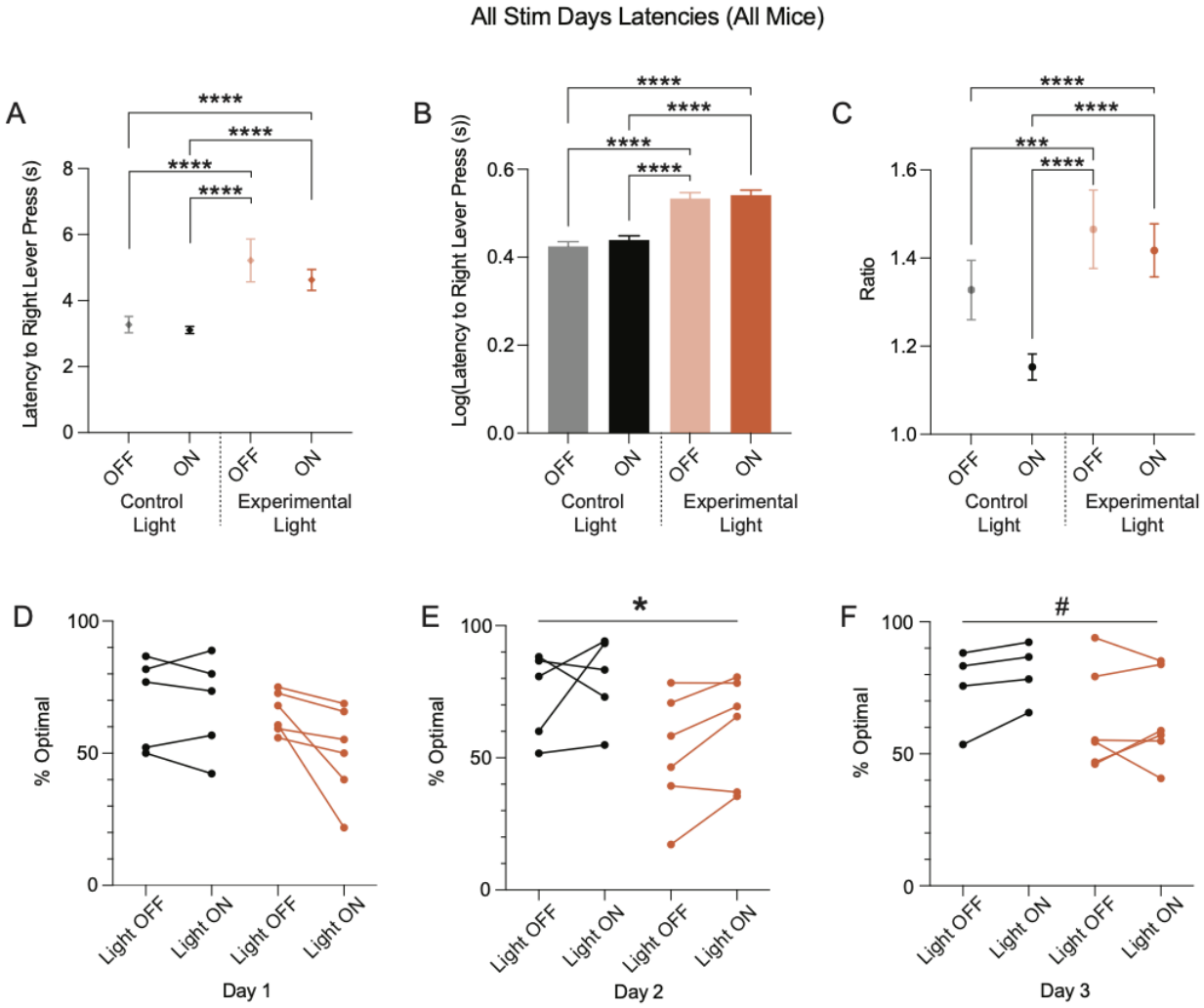

Figure 2.11: Inhibiting lOFC projecting MD neurons affects efficient performance on the chain instrumental task. (A-B) MD->lOFC inhibition's effects on latency to RLP (nonparametric and parametric tests, respectively, since latency data needs to be log transformed in order to follow a normal distribution) **(C)** MD->lOFC inhibition's effects on efficiency in performance. Ratio of 1 indicates that left seeking lever pressing was maximally efficient, i.e. left lever was only pressed as much as was needed to produce the right lever. **(D-F)** MD->lOFC inhibition's effects on percentage of optimal trials within a session (Stim Days 1-3). Optimal trials are defined as trials in which mice pressed the left seeking lever only as much as they needed to in order to produce the right lever. **** p<0.0001, *** p<0.001, * p<0.05, # p=0.07. Data points are mean+SEM.

Taken together, these findings suggest that the longer latency to RLP upon the production of the right lever in the experimental group did not result from motor impairments as mice were able to continue to press the left lever. Instead, it suggests that

this behavioral effect could be due to mice being less attuned to the production of the right lever as a consequence of blunted expectancy-related information conveyed in the MD-lOFC circuitry.

Discussion

We recorded and manipulated the activity patterns of the MD terminal population in lOFC during the learning and performance of an instrumental task with probabilistic and deterministic components. We found that MD-lOFC terminal population activity modulation around reward-proximal task epochs was sensitive to trial type as determined by the experience with the probabilistic action: Trials in which there were repeated reward-seeking actions performed, progressively building expectation (or in other words, increasing the expected value of the action), had greater MD-lOFC terminal population activity around the reward-proximal cue and following the reward-proximal deterministic action during reward consumption compared to trials in which the probabilistic action yielded the desired outcome after very few attempts. Inhibiting the activity of lOFC projecting MD neurons during this time period when the terminal population activity differentiated between these contrasting trial types affected animals' overall ability to efficiently perform the task, namely by increasing instances of perseverating on the reward-seeking lever rather than promptly responding to the emergence of the reward-proximal lever.

Cognitive flexibility, which is one of the main functions that MD-PFC circuitry is thought to serve (Rikhye et al., 2018), entails adapting one's responses in the face of changing response-outcome contingencies. With deterministic response-outcome

relationships, simple strategies can be used to obtain one's goal, but with probabilistic response-outcome relationships as in the case of our chain lever press task, integrating information from one's history with the task becomes important. Indeed, animals learn both from rewards received as a consequence of an action as well as their absence, which in the case of our task would translate to the consequences of accumulated left lever pressing not producing the right lever being important for learning. Especially at the outset of RR8 training, mice needed to modify their abstract representation of the task structure and their beliefs formed through their experiences with RR1-RR2-RR4 training: They had to learn through experience with the RR8 schedule that they had to on average press the reward-seeking lever more times than they previously had to (on average) in order to produce the reward-taking lever. By maintaining a dynamic representation of expectations, animals can interact with the world in an adaptive manner.

Previous work has shown that the OFC and the ventral tegmental area are important for learning from unexpected outcomes and adapting expectations and behavior accordingly (Takahashi et al., 2009). The results of our experiments suggest that outcome expectation in so far as it modulates current ongoing decisions and behavior could be influenced by MD's projections into the lOFC. Given the wealth of literature on OFC's role in updating cognitive maps and flexibly controlling goal-directed behavior, these findings provide an avenue through which to understand how OFC may be accomplishing these functions as a result of the cortico-striatal-thalamic-cortical loops it is embedded in. An interesting circuit node to study that could provide further insight into how expectation-related information may be conveyed via the MD-lOFC subcircuit is the ventral pallidum (VP). It has been known that one of the major sources of subcortical input

to MD is the VP (Ray & Price, 1993). Clinically, increases in VP-MD connectivity have been observed in medication-naïve patients diagnosed with schizophrenia, which is a neuropsychiatric disorder that the MD-PFC circuitry is heavily implicated in (Gong et al., 2019). Recently, it has been reported that trial-based reward prediction error (RPE) signals from the VP are more robust than RPE signals recorded in the nucleus accumbens, which in fact projects to VP (Ottenheimer et al., 2020). The VP may be the neural substrate that provides outcome history-based information to the MD, which in turn incorporates this information when providing updated prospective information (e.g. prediction) to OFC that can help guide future actions. In this regard, the MD could be viewed as sending a cognitive corollary discharge to the lOFC, which has been suggested to be a putative function of the MD higher-order thalamic nucleus (Mitchell et al., 2015)

One caveat to mention with our methodology is, given that we were measuring a bulk fluorescence signal, we were likely capturing heterogenous populations of MD-lOFC terminals, which we know synapse onto layers 1, 3, and 5/6 of cortex, both through our histology and published work on other MD-PFC circuits (Collins et al., 2018). Thus, we cannot determine to what extent expectation-related information may be getting relayed to corticocortical neurons vs. cortical output neurons in deeper layers of lOFC.

Author Contributions

E.A.Y.: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Visualization, Writing – original draft. C.M.G.: Conceptualization, Supervision, Funding Acquisition, Project Administration, Visualization. Writing – review and editing.

COI

The authors declare no competing interests.

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CHAPTER THREE: Orbitofrontal cortex projecting mediodorsal thalamic population's role in incentive learning

Abstract

A hallmark of flexible goal-directed behavior is not only to be able to learn contingencies that produce a certain outcome and make predictions based on this learning, but also to be able to modify behavior that leads to the outcome according to changes in the consequences of that behavior. Incentive learning is an example of a cognitive process by which outcome value is changed through experiencing the outcome in a different state, and this learning then becomes important for modifying behavior that typically results in that outcome. We sought to record and manipulate the activity of lateral orbitofrontal cortex (lOFC) projecting mediodorsal (MD) thalamic neurons during critical epochs for incentive learning – namely, when animals are actively registering a change in value of the primary reinforcer from instrumental training (i.e. sucrose reward), and when animals have to integrate this updated value of the primary reinforcer into their cognitive representation of the chain instrumental task and relate it to the secondary reinforcers therein (i.e. lever manipulanda). We found that licking-related activity of the MD-lOFC terminal population did not seem to reflect motivational state-induced value changes during reward re-exposure sessions. However, its endogenous activity during extinction test sessions when animals had to guide their action selection based on the learned change in reward value seemed to play a role in adjusting instrumental seeking actions following revaluation: Attenuating activity of MD-lOFC neurons during the test session blunted the expected change in instrumental seeking behavior following

revaluation. These experiments add to previous works that have investigated MD's role in outcome devaluation by 1) examining MD terminal activity locally in the lOFC, a region thought to be essential for updating outcome value, during reward re-exposure and 2) performing subcircuit specific and reversible manipulations during two different epochs of incentive learning.

Introduction

Goal-directed behavior is often defined as behavior that is sensitive to outcome devaluation as an operational way to distinguish it from habitual behavior or stimulus-response (Watson & de Wit, 2018). Outcome devaluation is a procedure that examines adaptively adjusting behavior following a change in the valuation of a contingent outcome. There are different methods of implementing outcome devaluation: Some experimenters utilize sensory specific satiation (i.e. specific to the outcome), others pair the outcome with an aversive experience; for example, lithium chloride injections. In spite of variations in the methodology for implementing outcome devaluation, the orbitofrontal cortex (OFC) has emerged as a brain region thought to be important for this cognitive process, even though there are ongoing debates regarding its importance for outcome devaluation in the context of instrumental vs. Pavlovian behaviors (Pickens et al., 2005, Parkes et al., 2018). Incentive learning in particular induces a change in the motivational state of the animal to accomplish outcome devaluation; crucially, the animal is then re-exposed to the outcome in its new motivational state to allow for value updating, and whether or not appropriate updating took place is subsequently gauged by the animal's propensity to perform the instrumental action learned to produce the outcome in a brief extinction test

session (Dickinson & Balleine, 1994). Our lab has demonstrated through chemogenetic manipulations that intact lateral OFC is critical for value updating during incentive learning following metabolic satiety (Baltz et al., 2018). However, it remains unclear how the OFC accomplishes this feat, and which circuit connections in the broader cortico-striatal-thalamo-cortical loops provide functional support. Given mediodorsal thalamus' (MD) striking connectivity with the OFC and the rest of the limbic circuit more broadly, we wanted to explore whether the MD-lOFC terminal population would be sensitive to reward consumption in differing motivational states, which is the crucial period for value updating through incentive learning (Vertes et al., 2015).

Motivational state changes prior to outcome re-exposure have been shown to more strongly influence instrumental responding distal to reward compared to proximal instrumental actions (Balleine et al., 1995). Thus, as an extension of the experiments described in Chapter 2, we conducted an incentive learning paradigm with the fiber photometry mice that had already learned the chain instrumental task. Furthermore, in a different set of mice, we chemogenetically manipulated the activity of lOFC projecting MD neurons during critical epochs for incentive learning. These experiments were conducted in an effort to better parse out which aspects of incentive learning the MD-lOFC subcircuit may be involved in.

Materials and Methods (in addition to those described in Chapter 2)

Animals

Mice were housed 2–5 per cage under a 14/10 hr light/dark cycle with access to food (Labdiet 5015) and water *ad libitum* unless stated otherwise. Mice were at least 5 weeks of age prior to intracranial injections and at least 7 weeks of age prior to behavioral training. All surgical and behavioral experiments were performed during the light portion of the cycle. The Animal Care and Use Committee of the University of California San Diego approved all experiments and experiments were conducted according to the NIH guidelines.

Negative incentive learning task

Following instrumental training as described in Chapter 2, the incentive value of sucrose reward was manipulated by maintaining food restriction (no shift in motivational state) or providing *ad libitum* access to home cage food (negative shift in motivational state) prior to an opportunity for sucrose revaluation in the operant context. For fiber photometry experiments, mice had *ad libitum* access to food and water ~1.5 - 2 hours immediately prior to the outcome revaluation session (same was true on subsequent test days). For all other experiments, mice received *ad libitum* access to food and water in their home cages overnight before the outcome revaluation session (same was true on subsequent test days).

Revaluation and testing sessions

Mice were maintained at their training food restricted state or their motivational state underwent a negative shift as described above. Mice were then maintained at their

assigned food restriction for all of testing. During the outcome revaluation session, mice were given re-exposure to sucrose during an RT120 session for 1 hour, with sucrose delivered on average every 2 minutes (or 1 minute for fiber photometry mice). The next day, mice were given a 5 minute (or 10 min for fiber photometry mice) non-rewarded test where responses on the left lever would produce the right lever on an RR8 schedule; however, right lever presses were unreinforced. In some experiments, the following day mice were given a longer rewarded test on an RR8 schedule.

Chemogenetic inhibition of MD-lOFC neurons

In order to target our chemogenetic manipulation to MD neurons projecting to lateral OFC (MD-lOFC neurons), we used a dual virus approach to express inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in a projection-specific manner. Male and female C57BL/6J mice received stereotaxically guided bilateral injections of rAAV5/hsyn-GFP-Cre (UNC Vector Core, ~300-400 nL) via Hamilton syringe into lOFC (coordinates from Bregma: A, +2.55 mm; M/L, 1.65 mm; V, 2.6 mm), as well as either Cre-dependent inhibitory DREADDs (AAV5/hSyn-DIO-hM4D(Gi)-mCherry, Addgene, ~250-400 nL) or a Cre-dependent control fluorophore (rAAV5/Flex-tdTomato, UNC Vector Core, ~250-400 nL) in MD (coordinates from Bregma: A, -1.34 mm; M/L, 1 mm; V, 3.6 mm at a 12° angle from ordinate for optimal targeting of MDm/c). ~30 minutes prior to the relevant behavioral session, mice were given an intraperitoneal injection of agonist Clozapine-n-oxide (CNO) (NIMH NDSP 10 ml/kg 1 mg/kg dose) in 0.9% isotonic saline based on our previous work looking at the *in vivo* time course of observing neural activity suppression with hM4D(Gi) (Gremel & Costa, 2013). After

experiments were completed, mice were euthanized, and their brains were extracted and fixed in 4% paraformaldehyde.

Results

Motivational state-dependent revaluation of instrumental outcome

In our negative incentive learning experiment, we induced a motivational state shift in mice through metabolic satiation, i.e. *ad libitum* access to food and water ~1.5 hours prior to an instrumental outcome re-exposure session. Following re-exposure to and consummatory experience with sucrose reward in the operant context mice were trained in, we examined how the propensity to press the left 'seeking' lever from the chain instrumental task mice were trained on was affected by this new learning (**Figure 3.1**).

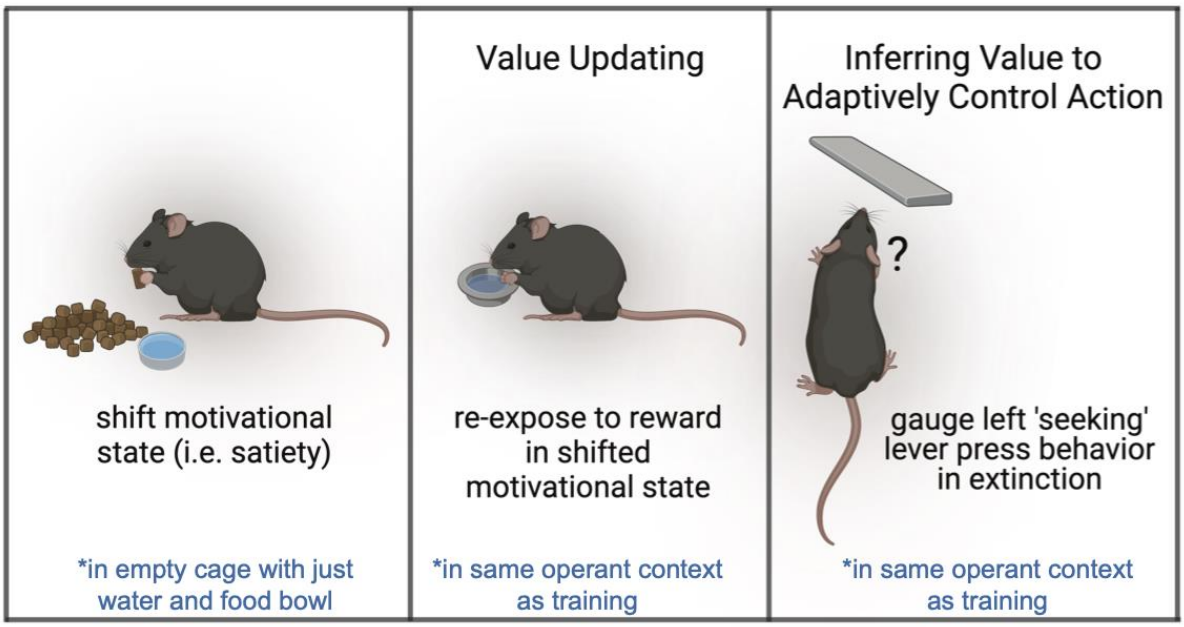

Figure 3.1: Schematic of motivational state-dependent negative incentive learning. In order for incentive learning to occur, the animal needs to re-experience the previously rewarding instrumental outcome in its new motivational state to update its value. If outcome value updating occurs as expected, in a subsequent extinction test session, animals will adapt their seeking lever press behavior to reflect their new valuation of the outcome in their shifted motivational state. Figure created with BioRender.

Before examining extinction test day performances, we wanted to ensure that mice were engaging with the prefeed and really shifting their motivational states. An indication that mice's motivational states were appropriately shifted is their within subject weight increase following the prefeed before the revalued re-exposure session (**Figure 3.2A**; Paired t test, $p<0.0007$). Secondly, the within subject decreases in number of licks and headentries made during the revalued re-exposure session relative to a control re-exposure session in which the mice's hunger states were not altered provide further evidence that the prefeed is sufficient to shift the motivational state of mice (**Figure 3.2B-C**; Paired t test, $p=0.0215$; Paired t test, $p=0.0060$).

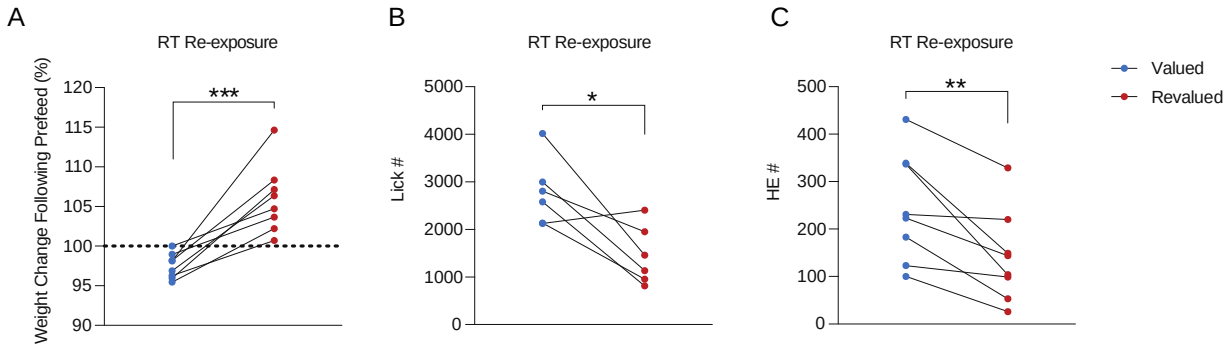

Figure 3.2: Prefeed before re-exposure session start is sufficient to shift the motivational state of mice. (A) Weights following prefeed divided by their weights before the start of the prefeed. For valued re-exposure sessions, the prefeed period just included water and no food, whereas for revalued re-exposure sessions there was both food and water available during the prefeed. **(B)** Number of licks emitted during the valued vs. revalued re-exposure sessions, **(C)** and number of headentries made during the two sessions. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

Given that mice showed evidence of shifting their motivational states and had an opportunity to update their valuation of the instrumental outcome, we were able to without confounds examine and interpret extinction test session performances from the day following the reward re-exposure session. Similar to before the start of the revalued re-

exposure sessions, mice had increased weights at the end of the prefeed before the re-valued extinction test sessions (**Figure 3.3A**; Paired t test, p=0.0003). As expected, mice were less motivated to press the reward-seeking lever on the revalued test day following the revalued re-exposure session (**Figure 3.3B**; Paired t test, p=0.0301). Of note, test day left lever pressing is assessed relative to press rates during training when mice were food restricted and highly motivated to seek the sucrose reward. Together this data indicate that mice were able to successfully update their valuation of the instrumental outcome, and use this updated value to adapt their instrumental action in the absence of reward information.

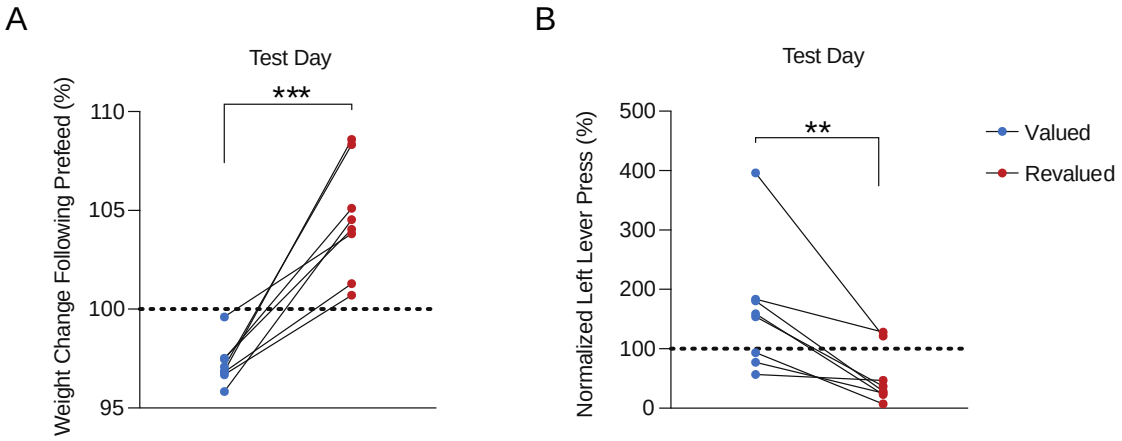

Figure 3.3: Extinction test day performances following revalued vs. control (valued) re-exposure sessions. (A) Weight changes at the end of prefeed before the start of the extinction test sessions. **(B)** Left seeking lever press rate relative to RR8 training LLP rates. Extinction test sessions were also conducted under the RR8 schedule, but with no reward delivery. *** p<0.001, ** p<0.01.

MD-lOFC terminal population activity during reward consumption does not seem to reflect value change induced by a motivational-state shift

While previous work from our lab has demonstrated that intact OFC function is important for appropriate value updating (Baltz et al., 2018), MD-lOFC inputs' role in

incentive learning has remained unexplored. One possibility is that, during reward re-exposure, the different consummatory experience based on motivational state is reflected in the MD-lOFC terminal population activity. Depending on the direction of the motivational shift (i.e. less vs. more hungry), perhaps the direction of value updating (i.e. decrease vs. increase) could be influenced and/or encoded by the MD-lOFC terminal population if it carries state-related information, for example. To explore these possibilities, we recorded MD-lOFC terminal population activity using fiber photometry during reward re-exposure sessions. As the re-exposure sessions basically consist of randomly timed deliveries of sucrose reward, the main behaviors mice perform are headentries (i.e. reward port checking) and licking (i.e. reward consumption). When we examined MD-lOFC terminal population activity surrounding first lick after reward delivery, we did not observe any meaningful statistically significant differences between activity traces from revalued vs. valued re-exposure sessions (**Figure 3.4A**). In addition, peak activity modulations from peri-event calcium activity traces surrounding licking following reward delivery did not differ between valued vs. revalued re-exposure days (**Figure 3.4B;** Paired t test, p=0.5889). Since extinction test performance is the behavioral read out for how much mice value the instrumental outcome and are motivated to seek it, we wanted to explore the possibility that the varying degree to which mice changed their test day performance across valued and revalued sessions may be related to MD-lOFC terminal population activity during re-exposure sessions. However, the correlation between peak activity modulation difference scores from valued vs. revalued re-exposure sessions and test day performance difference scores from valued vs. revalued extinction test sessions was not significant (**Figure 3.4C**). Taken together, these data suggest that

on a gross level, the MD-lOFC terminal population activity does not reflect instrumental outcome value during incentive learning per se, but this thalamic input could still play a supportive role in value updating processes taking place in the cortex proper in lOFC.

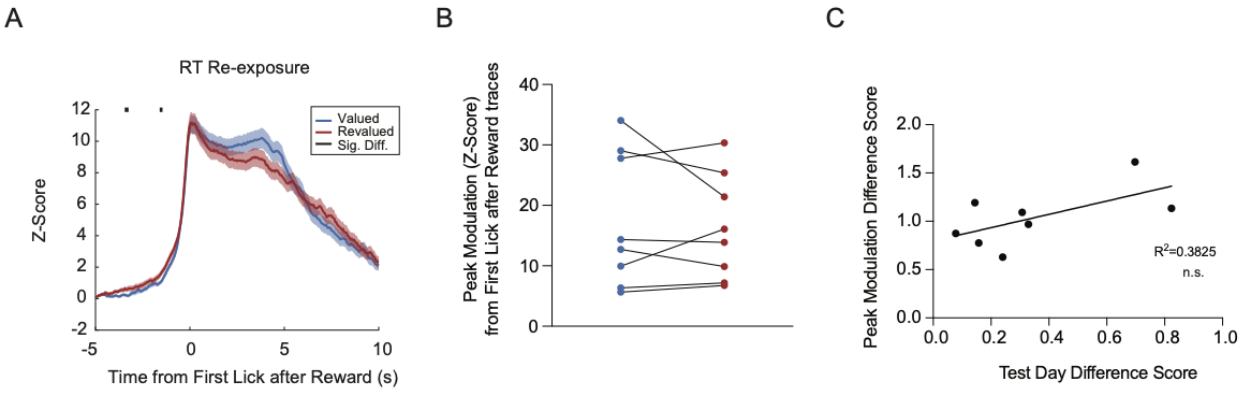

Figure 3.4: MD-lOFC terminal population licking activity after reward delivery during revalued vs. valued re-exposure sessions. (A) Licking peri-event calcium activity relative to baseline during reward re-exposure sessions (permutation tests, 10,000 random permutations, α = 0.05 for valued vs. revalued comparisons) **(B)** Peak MD-lOFC terminal population activity modulation (i.e. average of 4 consecutive highest Z-Score values) within the time frame depicted in 3.4A across valued vs. revalued re-exposure sessions. **(C).** Pearson's correlation between peak modulation differences across valued and revalued re-exposure sessions and differences across valued and revalued test day performances (not significant).

Chemogenetic attenuation of lOFC projecting MD neurons during critical epochs for incentive learning affects adaptive behavior on test day

We identified two key epochs during which MD-lOFC input could be important for appropriate value updating and/or adaptive use of the updated value (**Figure 3.5**): The first is the one-hour instrumental outcome re-exposure session when mice are sampling the sucrose reward in their new motivational state, and the second is the extinction test session when mice have to apply their updated valuation of the sucrose reward to their action selection in the instrumental task context. We thus had two separate experimental groups that underwent activity manipulations at one of the two epochs.

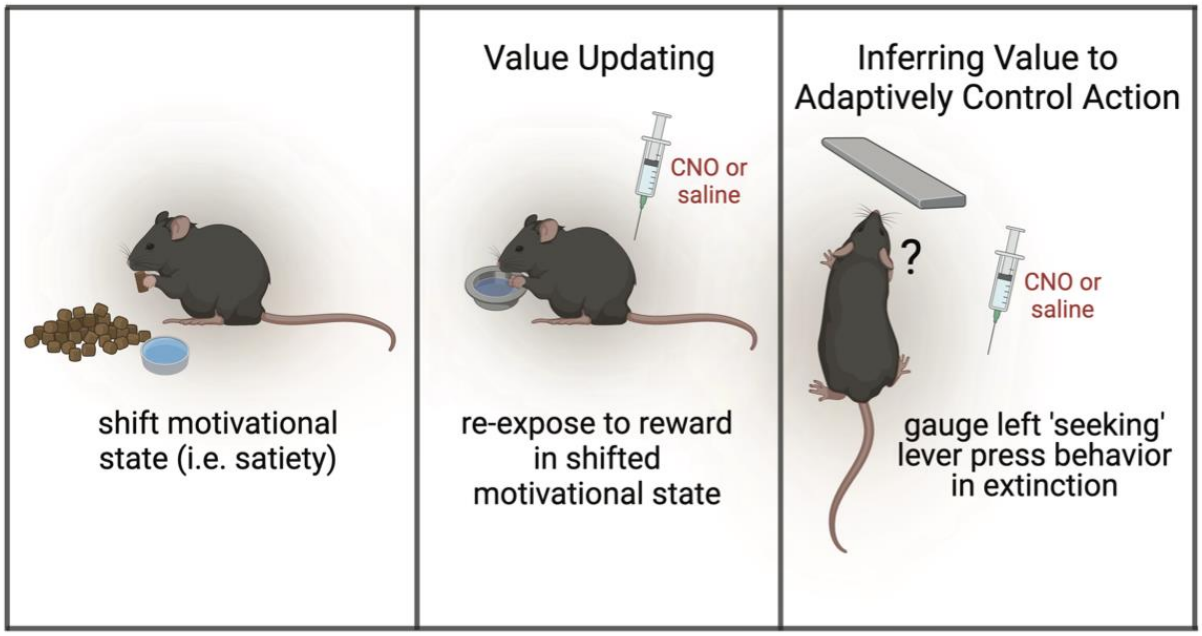

Figure 3.5: Schematic of critical epochs for incentive learning when lOFC projecting MD neurons are chemogenetically attenuated. CNO, which is the designer drug that activates the inhibitory DREADDs (H4) expressed in MD-lOFC neurons, can be administered 30 minutes prior to either the reward re-exposure session (value updating) or the extinction test session (use of updated value). Figure created with BioRender.

We opted to use a chemogenetic approach to attenuate the activity of lOFC projecting MD neurons given the extended nature of the re-exposure session. The dual-virus strategy described in Chapter 2 was effective in achieving Cre-dependent inhibitory DREADD (H4) expression in MD-lOFC neurons. No meaningful group differences or group by training day interactions were observed during training in the control and experimental groups (**Figure 3.6**).

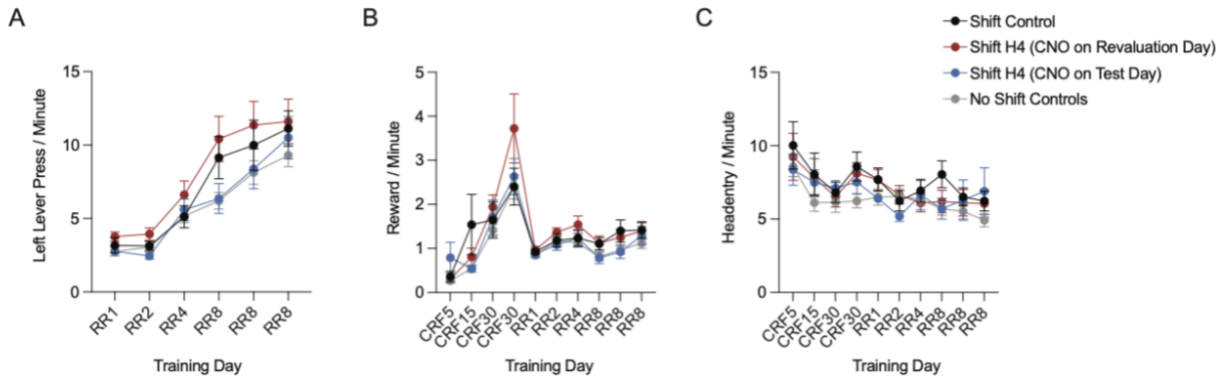

Figure 3.6: Instrumental training performances of control and experimental groups. (A) Left seeking lever press rate (2-way ANOVA Group $F(3,35)=2.264$, $p=0.0981$, Interaction $F(15,175)=1.388$, $p=0.1573$), **(B)** reward / right taking lever press rate (2-way ANOVA Group $F(3,35)=3.159$, $p=0.0367$, no significant differences from multiple comparisons, Interaction $F(27,315)=1.317$, $p=0.1385$) **(C)** and headentry rate across training (2-way ANOVA Group $F(3,35)=1.060$, $p=0.3787$, Interaction $F(27,315)=0.8602$, $p=0.6695$). Data points are mean+SEM.

There were also no significant group differences in the licking behavior mice exhibited during the reward re-exposure session (**Figure 3.7A-B**; 1-way ANOVA $F(3,34)=2.559$, $p=0.0712$; 1-way ANOVA $F(3,35)=2.649$, $p=0.0640$). Thus, any test day performance differences between experimental and control groups cannot be due to differences in reward sampling during the re-exposure session. When we examined extinction test day performance across groups, as expected, compared to groups in which mice shifted their motivational state prior to revaluation and extinction test, the control groups in which mice did not shift their motivational state performed significantly more left seeking lever presses normalized to RR8 training LLP rates, irrespective of whether they expressed inhibitory DREADDs activated by CNO during reward re-exposure (**Figure 3.7C**; Brown-Forsythe ANOVA $F(3,21.60)=19.69$, $p<0.0001$). Interestingly, the shifted experimental group in which mice received a saline injection before reward re-exposure and a CNO injection before the extinction test performed significantly more left seeking lever pressed normalized to training compared to the shifted control group in which mice simply

expressed a red fluorescent protein in MD-lOFC neurons instead of inhibitory DREADDs. These results suggest that attenuating the activity of lOFC projecting MD neurons during the extinction test when mice have to use updated reward value to adjust their instrumental seeking actions blunts the typical decrease in left lever press rate normalized to training, but not to a degree that results in LLP rates that match training rates. Taken together with the finding that attenuation of MD-lOFC neurons during value updating did not significantly alter the typical reduction in left seeking lever presses following negative incentive learning, we conclude that the MD-lOFC subcircuit may serve a more important role in integrating updated outcome values into the animal's internal task representation in order for task performance to fully reflect current outcome valuation.

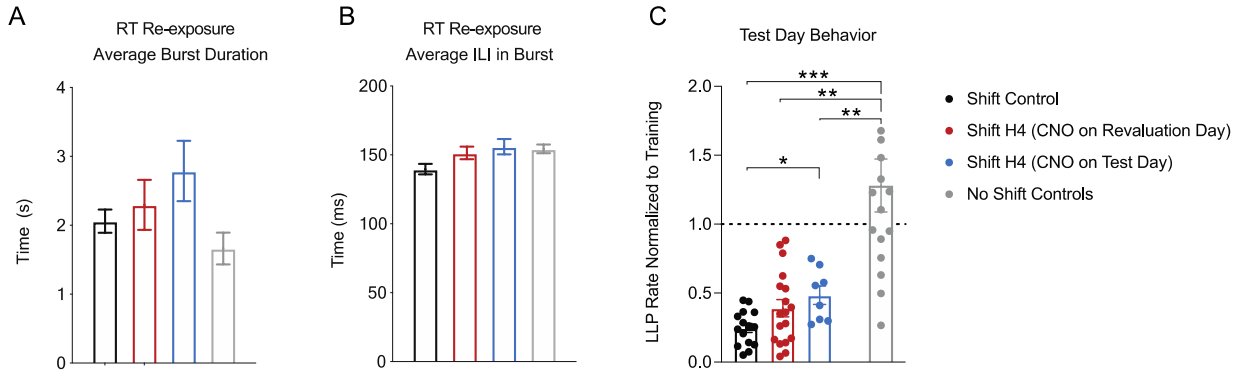

Figure 3.7: Re-exposure and extinction test day behavior across control and experimental groups. (A) Lick burst durations during reward re-exposure session. **(B)** Average interlick interval within lick bursts exhibited during reward re-exposure session. **(C)**. Left seeking lever press rate relative to RR8 training LLP rates. Extinction test sessions were also conducted under the RR8 schedule, but with no reward delivery. *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Data points are mean+SEM.

Discussion

Motivational states – particularly primary motivational states such as hunger – modulate hedonic response to an outcome upon contact (Berridge et al., 1984). This is

why it is crucial for animals to have direct experience with an outcome in order for them to learn about its change in value due to an altered state. We observed relatively high levels of activity modulation in the MD-lOFC terminal population aligned with licking behavior following reward delivery during outcome re-exposure sessions for incentive learning. Given that consummatory contact with the outcome and the resulting response are thought to be when value assignment is taking place, it is interesting that we did not see evidence of activity differences in the MD-lOFC terminal population depending on the direction of the shift in motivational state prior to re-exposure.

One explanation is that, rather than conveying information such as current value, these thalamic inputs may instead be influencing cortical ensembles in lOFC that maintain current outcome value. There have been reports of MD enhancing lateral connectivity of prefrontal cortex (PFC) neurons in an attentional control task (Schmitt et al, 2017). In these experiments, PFC populations were found to represent task rules dictating which sensory modality to pay attention to, and in contrast, the MD seemed to play a more supportive role in enhancing functional connectivity within these PFC ensembles depending upon the current rule. While the cortical region in question in this study was the prelimbic cortex, an analogous function could be served by MD-lOFC inputs during a decision-making task that reportedly requires the OFC. The interpretation that the MD-lOFC terminal population is influencing local functional connectivity in the lOFC during reward re-exposure / outcome value updating could be extended to test day when mice have to integrate the updated outcome value with their internal model of the task. Considering that the OFC is viewed as the locus of the cognitive map, there is surprisingly little known about how thalamic and other long-range subcortical inputs may be

contributing to updating task representations that are thought to underlie flexible goal-directed behavior.

We know from unpublished work in our lab and experiments conducted on MD projections into medial PFC that the MD can modulate cortical excitation-inhibition balance as well as the temporal integration window for neural firing through its functional connections with both local excitatory and (primarily parvalbumin-positive) inhibitory neurons in the PFC (Delevich et al., 2015). Thus, the relative increase in MD-lOFC terminal population activity we observed during licking behavior in the context of outcome value updating could also be reflective of thalamic engagement that modulates cortical gain. Future studies would ideally simultaneously record activity from MD terminals in lOFC in conjunction with OFC pyramidal neurons at a finer timescale than what the fiber photometry technique allows.

Author Contributions

E.A.Y.: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Visualization, Writing – original draft. C.M.G.: Conceptualization, Supervision, Funding Acquisition, Project Administration, Visualization. Writing – review and editing.

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The authors declare no competing interests.

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