Flexibility & Adaptability: The Role of the Lateral Habenula & Medial Prefrontal Cortex

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Abstract

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The ability to make appropriate decisions that result in an optimal outcome is critical for survival, yet the same processes that support flexibility can also give rise to maladaptive choices when they break down. Identifying the neural circuits that mediate flexible behavior, and how those circuits are affected in maladaptive states, is invaluable to our understanding and subsequent treatment of psychiatric conditions. The lateral habenula (LHb) plays a well-known role in reward and aversive processing, but accumulating evidence from our laboratory and others has implicated the LHb in flexible decision-making more broadly (Baker et al., 2015; Mizumori & Baker, 2017; Hones & Mizumori, 2022; Ahmadlou et al, 2025). Flexible behavior recruits a number of cognitive processes including contextual memory and motivational state information that the LHb does not directly mediate, raising the question of which broader circuits work together with the LHb to support adaptive choices. Converging evidence points

toward a circuit in which contextual information from the hippocampus (HPC) reaches the LHb via the medial prefrontal cortex (mPFC), where the LHb integrates that information with motivational state signals to enable strategy switching when goals or contexts change (Hones & Mizumori, 2022). This same HPC–mPFC–LHb circuit is implicated across a variety of psychiatric conditions characterized by maladaptive decision-making, including opioid use disorder, where morphine withdrawal disrupts mPFC function at least in part through serotonin depletion and pyramidal cell hyperactivity (Goeldner et al., 2011; Piao et al., 2017). Serotonergic agonists such as psilocybin have the potential to act on this circuit by promoting dendritic growth and other markers of plasticity (Shao et al., 2021; Pacheco et al., 2023), offering a potential therapeutic avenue for restoring flexibility in maladaptive states.

To test the role of this circuit in flexible and adaptive behavior, we approached the problem from two complementary directions. First, we asked how disrupting a key node of the circuit, the LHb, shapes flexible and adaptive behavior on a spatial set-shifting task. Pharmacologically inactivating the LHb with muscimol did not abolish flexibility *per se*: rats still readily changed their choice patterns from trial to trial, even at more elevated levels than saline controls. Inactivation did, however, impair their ability to direct that flexibility toward a new, correct strategy after uncued shifts in reward contingencies, indicating that the LHb specifically supports the adaptive use of negative outcome information to guide subsequent decisions. As such, without an intact LHb, flexibility manifests as maladaptive choice rather than effective strategy switching.

Next, we approached the same circuit from the opposite direction by inducing a maladaptive state, morphine withdrawal, and asking how that state alters circuit function, focusing on the mPFC. We combined a spatial set-shifting task with a novel object recognition test (NORT) and recorded prelimbic neurons in the mPFC *in vivo* using calcium imaging. Morphine-withdrawn rats showed impairments across several aspects of flexible behavior on the

set-shifting task, as well as deficits in novel object recognition. At the neural level, prelimbic neurons in morphine-withdrawn rats were hyperactive, but less active in response to novel objects, and this inhibited neural response was consistent with their behavioral NORT impairment. Functional clustering of prelimbic responses showed that distinct subpopulations of neurons changed their activity selectively depending on the drug condition, indicating that maladaptive states reshape the intrinsic activity patterns of subpopulations of prelimbic neurons. A single therapeutic dose of psilocybin (1 mg/kg) alleviated some aspects of the morphine-related impairments on both the set-shifting task and the NORT, and further improved set-shifting performance in saline-treated control rats. The behavioral effect of psilocybin treatment was paired with improved neural activity in prelimbic neurons across testing conditions, as well as during the NORT. This suggested that serotonergic plasticity has a broader, positive effect on cognition that may extend beyond pathological states.

Together, these findings contribute to our understanding of the HPC–mPFC–LHb circuit as a coordinated system that uses context, outcome, and internal state information to guide flexible and adaptive behavior. Importantly, our results highlight a broader issue in the flexibility literature, where flexibility is often inferred from task outcomes alone. As Searle's Chinese Room illustrates, observing the output of a process does not reveal the process itself. When we examined trial-to-trial strategy use rather than just the outcome alone, we found that disrupting different nodes of this circuit produced hyperflexible, maladaptive states rather than the hypoflexibility that outcome-based measures alone would suggest. We also identify psilocybin as a potential intervention that can restore, and potentially enhance, the behavioral flexibility and adaptability this circuit typically supports.

Table of Contents

List of Figures

Acknowledgements

My interest in the brain came about a fairly traditional way—through classes that I took at Bellevue College and Seattle University. The initial draw was a fascination with the circumstances and life experiences, as well as biological makeup, that lead individuals to make the decisions that they do. Of most interest were decisions that are met with negative consequences, as is so often seen in addiction. Professor Eric Davis at Bellevue College, as well as Dr Michael Spinetta at Seattle University, led wonderful courses that deepened my fascination with human behavior, which ultimately shaped my future choices in both education and career. During my time at Seattle University, I was lucky to join the HARC research team led by Dr Molly Brown out of DePaul University, in Chicago, to research and understand the social support networks of individuals experiencing chronic homelessness. The field work was incredibly insightful and only strengthened my pull towards understanding the brain.

That pull eventually led me to the Mizumori lab at the University of Washington, where the brain could be studied directly. I had not previously understood that the brain could be manipulated with such precision, or that behavior itself could be shaped and studied at that level of detail. Everything about the Mizumori lab felt new and exciting to me. I spent a great deal of that time alongside Dr Kevan Kidder, a graduate student at the time, trying to absorb as much as I could from his approach to research. He was incredibly generous with his time, staying well past the end of his day simply to walk me through new techniques of skills, and a lot of my early scientific foundation was thanks to him.

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not think I would have completed the program without her support. Beyond that support, Sheri has a steadfast approach to research that I have inherited, which is very important in this line of work, as it turns out. No matter how many times someone tells you to be persistent in your research, it doesn't hit home until you are trying out an experiment after a third fail. Research has a way of humbling you, and I appreciate that.

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I also did not expect to find as much joy in teaching as I did. The students at the UW are so thoughtful and kind and I am grateful to have taught, and to have learned how to teach, alongside them. A number of those same students went on to join my research and contribute directly to my experiments, which has meant more to me than I think they realize. Training rats for just one of my experiments required roughly 1,280 hours of hands-on work, and this figure does not include the additional time spent on husbandry, tissue processing, or the countless hours students spent simply learning and rehearsing new skills in the lab. The day-to-day care

and training of our animals falls primarily to a handful of extraordinary undergraduate researchers, each of whom has chosen to dedicate their time to advancing our understanding of the brain and behavior. My work, like the majority of research across this field, depends heavily on the labor that undergraduate students provide, and I am deeply grateful to each student who chose our lab, chose to work alongside me on these experiments, and made my time in this program such a joyful one.

I would also like to add a word of gratitude to the animals who made this research possible at all. Working with rats throughout my projects gave me an immense appreciation for animal research. I am consistently impressed by their ability to learn complex tasks, to adapt, and to navigate the demands we placed on them. I feel a great deal of empathy for them, and I am genuinely grateful for what they taught me.

And, of course, I owe an enormous debt of gratitude to my family. My father immigrated to the United States from Russia and spent 5 years building a life and livelihood here before my mother and I were able to join him. My parents sacrificed a great deal so that we could be here, and so that I could pursue the education I have, and I do not have adequate words for how grateful I am for that sacrifice. My husband, Ed, whom I met during my time at Seattle University, has weathered every high and low this program had to offer alongside me. I am endlessly grateful for his support throughout this entire process—there really needs to be an honorary degree reserved for the partners of graduate students; he truly deserves it. Lastly, I would like to thank my furry family: my dogs, Charlie and Aaka, and my cat, Yozhik, for pulling me out of the lab and out of the house in ways that did more for my well-being than I recognized at the time.

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Chapter 1

Response Flexibility: The Role of the Lateral Habenula & Medial Prefrontal Cortex

Hones & Mizumori, 2022.

Introduction

One's ability to behave intentionally, especially when presented with options, involves a number of complex processes such as selectively attending to relevant sensory input, determining whether environmental context conditions have changed from what is expected based on past experience, selecting an appropriate action, assessing the outcome of the selected action relative to internal state information, and then updating one's knowledge about the context and response outcomes to be prepared for the next encounter. A common driver of all of these processes is not only one's memory but also the ability of information about one's internal state to modulate the efficiency of memory processing and thus memory's impact on subsequent behaviors. Therefore, in order to fully understand real world goal-directed and flexible behavioral adaptation, it is necessary to understand not only how the brain processes new information to form memories, but it is essential to clarify how internal state (i.e. motivation) information comes to regulate the behavioral implementation of context memories.

There are a number of excellent and detailed reviews on brain mechanisms of context memory (e.g. Burgess et a., 2002; Eichenbaum, 2017; Lisman et al., 2017; Maurer & Nadel, 2021). Since the hippocampus (HPC) is known to be critical for context memory, and since hippocampal neurons are sensitive to a broad range of external and internal sensory information (including rewards and aversive stimuli), our definition of 'context' extends beyond the external sensory environment. While we have made exciting and significant advances in our understanding of the molecular, as well as neural circuit and systems changes during context

learning and memory, precisely how context memory intersects with information about one's current motivational state to promote adaptive behavioral outcomes is not clear. This is an important problem to solve for it applies to many of our everyday behaviors and decisions. As an example, while you may have learned about and understand the health benefits of exercise, your motivational state may conflict with this knowledge, resulting in you deciding not to go to the gym.

Here, we focus specifically on the issue of how brain systems that integrate context memory and motivation information come to enable freely-navigating rats to quickly switch behaviors by flexibly responding to changes in environmental conditions. One approach to resolving this issue is to consider the advantage that cortical evolution may have conferred onto a pre-existing, evolutionarily conserved experience-dependent response flexibility brain system that involves the epithalamic structure, the habenula. In fish, amphibians, reptiles, and birds, the habenula receives sensory and internal state information while sending strong signals to midbrain structures that regulate overt actions (Stephenson-Jones et al., 2012). A critical role of the habenula in response flexibility is supported by findings that habenula disruption leads to impaired approach or avoidance responses to dynamic shifts in internal conditions or information, such as hormone levels (Ogawa et al., 2021), rules for task performance (Palumbo et al., 2020), or external environmental context cues. As the neocortex evolved in mammals, so did the prominence of the lateral division of the habenula (the lateral habenula, or LHb) as well as LHb connectivity with frontal cortical areas. Thus, it has been hypothesized that while the mammalian LHb continues to support flexible responding, it does so based not only on sensory and internal state input, but also frontal cortical (presumably context memory) input (Mizumori & Baker, 2017). Given the growing number of excellent and relevant published review articles, in the following sections, we only briefly highlight key findings that support the claim that the LHb plays important roles in context memory through its interactions with the medial prefrontal

cortex (mPFC), in processing motivation information, integrating context memory and motivational state information, and in response flexibility. Then we suggest a novel hypothesis to address the unanswered question of how the LHb, specifically, enables adaptive context-dependent response flexibility.

LHb is a hub for memory and internal state information

At any given point in time, the current constellation of neural activity and functional connectivity across the brain (considered here as the internal state) defines the neural foundation within which new information is processed. One often refers to such foundational neural states relative to a particular functional attribute such as a cognitive state, motivational state, and/or behavioral/response state. As an example, the patterns of cortical neural activity that exist prior to stimulus exposure (reflecting the cognitive state) effectively determines how new sensory information is processed and perceived, which in turn defines our interpretation and interactions with the world. In everyday life, different neural states do not function independently, but rather they are interdependent. For example, it is well known that altered motivational states, such as that which occurs when stressed, can bias the efficiency of information processing to improve (or impair) memory. The LHb presumably at least identifies, if not also retains (Andalman et al., 2019), current cognitive and motivational state information in order to adjust behavioral responding as task conditions change.

Cognitive State: Context Memory

Significant evidence supports the generally-accepted view that the HPC is critically important for context memories (e.g. Burgess et al., 2002; Eichenbaum, 2014; Howard & Eichenbaum, 2013; Place et al., 2016; Smith et al., 2013; Maurer & Nadel, 2021). Many studies have shown that place cells in the HPC fire strongly when an animal occupies a particular location (a place field; O'Keefe & Nadel, 1978; Gothard et al., 1996). When almost any feature of

the context changes, HPC place fields remap, or change their spatial and temporal patterns of firing. Additionally, place fields link to represent recent past, present and future context information (e.g Muller & Kubie, 1987; Wilson & McNaughton, 1993; Mizumori et al., 1999; Ferbinteanu & Shapiro, 2003; Leutgeb J et al., 2005; Leutgeb S et al., 2005; Smith & Mizumori, 2006; Nakashiba et al., 2009). In the natural world, it would be unfavorable for an animal to explore an environment similar to one that they have explored in the past that led to danger. Thus, when faced with a new but similar context, the HPC is thought to retrieve information about similar previous experiences (Spiers et al., 2015), then evaluate the extent to which the current context varies from the retrieved (expected) context (Mizumori et al., 1999; Vinogradova, 2001). The degree of similarity between expected and current context information seems reflected in HPC output signals (Mizumori, 2013). Without proper functioning of the HPC, context memory-guided behavior becomes significantly impaired (e.g. Morris et al., 1982; Gridchyn et al., 2020).

LHb plays a role in context memory

Early studies that probed LHb functions discovered that its inactivation results in analgesic-like effects at the time of tonic pain presentation (Fuchs & Cox, 1993). Soon after, it was shown that electrical stimulation of the LHb resulted in aversive behaviors perhaps by generating an aversive signal (Matsumoto & Hikosaka, 2009a; Friedman et al., 2011). Indeed, LHb terminals in the ventral tegmental area (VTA) and rostromedial tegmentum (RMTg) mediate behavioral avoidance (Lammel, 2012; Stamatakis & Stuber, 2012). Therefore, LHb is often considered the 'aversion center' of the brain (Baker et al., 2016). As a result of more recent studies, however, a number of functions are now attributed to the LHb in addition to the initial idea that it serves to provide an aversive signal. For instance, Congiu (2019) found that aversive foot shocks not only excite the majority of LHb neurons, but they also inhibit the activity of a small population of excitatory LHb neurons, indicating a more complex function of the

structure. Also, neurons in the LHb have been found to exhibit changes in activity patterns to rewards, suggesting the LHb contributes to signaling information to both aversive and rewarding stimuli (Matsumoto & Hikosaka, 2009a). An important role of the LHb in HPC-related context memory has been suggested in numerous studies. For example, LHb inactivation results in disruption of memory retrieval as well as an inability to update the spatial configuration of the environment (Mathis, 2015). LHb neurons have also been shown to keep track of choice outcomes, implicating it in memory for decisions made during goal-directed tasks (Baker et al., 2015; Kawai et al., 2015). LHb, then, appears to participate in the signaling of memories in the recent past, as well as memories collected over longer periods, suggesting a perhaps more general modulatory role in memory (Bromberg-Martin et al., 2010).

Numerous studies more explicitly show that the LHb is necessary for accurate context memory processing since its disruption impairs HPC-dependent context memory tasks such as the spatial delayed alternation and probabilistic reversal maze tasks (Baker et al., 2015, 2017, 2019). LHb is also involved in tasks such as novel object recognition (Goutagny et al., 2013) and the water maze task (Thornton & Davies, 1991; Lecourtier et al., 2004). It has also been shown that the spiking of LHb neurons aligns with HPC theta phase, suggesting a crucial interaction between the two regions (Aizawa et al., 2013). In the case of context-dependent fear memory, which necessitates the association between a context and an aversive cue presentation, inactivation of the LHb impaired the ability to appropriately respond to an aversive context (Durieux et al., 2020). In sum, a role for the LHb in context memory seems clear.

mPFC as a critical node for context memory and flexible behavior

An important outstanding question is the source of context information for the LHb. It is possible that the HPC relays contextual information to the LHb to guide proper behavioral responses. There are, however, no known direct connections between the HPC and the LHb,

suggesting the involvement of an intermediary brain region. Many studies have demonstrated the importance of the communication between the cortex and HPC in context memory and response flexibility (Avigan et al., 2020; Spellman et al., 2015; Tamura et al., 2017). Notably, one cortical brain region that plays a crucial role in action selection when responding to changing contexts, as well as receives strong synaptic innervation from the HPC, is the medial prefrontal cortex (mPFC) (Gilmartin & Helmstetter, 2010). When the infralimbic and prelimbic areas of the mPFC are inactivated, rats exhibit a significantly higher escape latency if the escape platform in a water maze is shifted to a different area of the maze compared to controls (de Bruin et al., 1994; Haddon & Killcross, 2011). Some mPFC neurons are preferentially active during specific behavioral states, giving rise to possible state information that the LHb can use to influence behavior (Halladay & Blair, 2015). Dysfunction of either the HPC or the mPFC often result in similar context memory and decision-making impairments, but several studies reveal that they make distinct contributions. The HPC plays a greater role in the formation and retrieval of memories about spatial contexts (O'Keefe & Nadel, 1978; Maurer & Nadel, 2021), while the mPFC plays a more crucial role in retrieval of distant memories that are generalizable to similar contexts (DeNardo et al., 2019; Samborska et al., 2021).

mPFC neurons display neuronal activity comparable to that of the LHb in that specific subpopulations display different activity in response to appetitive and aversive stimuli (Rubio et al., 2019; Capuzzo & Floresco 2020). This suggests that the mPFC and LHb may have common functions and/or they both participate in behavioral flexibility. Such findings imply a potentially important interaction between the mPFC and LHb. Indeed, studies using both anterograde and retrograde tracers have identified mPFC fibers terminating at the LHb, suggesting a functional connection (Kim & Lee, 2012; Mathis et al., 2021). What might be the functions of mPFC-LHb connections? With the apparent co-evolution of the LHb and neocortex came the ability to have greater intentional control over the execution of behaviors. mPFC signals in particular, become

a strong candidate intermediary structure to integrate the present HPC-derived context information with existing memories, and then pass response options to subcortical systems to determine which adaptive response is appropriate for the current motivational state (discussed in more detail below; Hones & Mizumori, 2022). Recent studies have attempted to uncover the nature of the signal from the mPFC to the LHb as well as its impact on behavioral output. Mathis and colleagues (2021) conducted a sequence of experiments characterizing the activity pattern of mPFC neurons that project to the LHb and their role in stress. They found that mPFC cells send signals to the LHb in the presence of a stressful event such as a foot shock. Moreover, the LHb cells that received signals from the mPFC had differential impacts on behavioral output based on the different network projection profiles. For instance, the mPFC-LHb-locus coeruleus projection played a major role in cocaine seeking, implicating this projection in reward-seeking behavior. The mPFC-LHb-raphe projection was implicated in freezing behavior in response to a stressor. Interestingly, these findings are consistent with the theory that the mPFC relays context-specific information to the LHb, which can serve as a brake signal (Sleezer et al., 2021) to cease behaviors when appropriate, or to engage behaviors in other aversive contexts.

In further support of the hypothesis that contextual information is relayed to the LHb, the prelimbic region of the mPFC (an area to projects to the LHb) has been shown to be necessary in flexible decision-making on hippocampal-dependent tasks (Kidder et al., 2024). Prelimbic neurons within the mPFC have been shown to encode task rules and reward outcomes that support animals as they adapt to new task contingencies (Sackett et al., 2019; Spring et al., 2021). Given that the mPFC receives dense projections from the hippocampus (Hoover & Vertes, 2007), contextual information can reach the mPFC to help guide flexible and appropriate choices. In support of this interpretation, Sánchez-Bellot and colleagues (2022) showed that there are two hippocampal pathways that project to the mPFC, and the types of neurons they synapse onto in the mPFC guides approach or avoidance. This suggests that the hippocampus

can inform different mPFC subpopulations to guide appropriate behavior (i.e. approach or avoidance). Thus, disruptions of the mPFC would be expected to impair the contextual information transfer from the hippocampus to the LHb.

Another potential source of context information for the LHb is the septal complex. The septum is a subcortical midline structure that is divided into medial and lateral septal portions (MS and LS, respectively) each of which have strong connections with the HPC and LHb (Swanson & Cowan, 1979). Inactivation of the MS results in spatial working memory deficits, impairments in HPC place cell activity, as well as impairments in processing contextual information, implicating the septum in processing contextual memory (Mizumori et al., 1989; Leutgeb & Mizumori, 1999; McGlinchey & Aston-Jones, 2018). Separating the functional contributions of the LS and MS to LHb has been challenging. The LS receives input primarily from the HPC, and projects to the MS, which projects back to the HPC via the fornix. A strong output of both the MS and LS is to the LHb (for an in-depth review of septal inputs and outputs, see Swanson & Cowan, 1979). Silencing either the LS or the MS results in overall decrease in avoidance behavior, while stimulation of excitatory MS inputs to the LHb induced conditioned place aversion in a two-chamber avoidance task (Veening et al., 2009; Zhang et al., 2018). The aversion-induced behavioral effect was positively correlated with stimulation frequency, which suggests that MS inputs play a significant role in driving aversive behavior in response to an aversive context. In terms of septal influences on context memory, the MS and LS are thought to play a role in maintaining HPC theta that is necessary for proper spatial and contextual memory integration (Tsanov, 2018). While the MS has reciprocal connections with the HPC, the LS only receives unidirectional inputs from the HPC (Tsanov, 2018). Thus, the MS may be driving both LHb and HPC activity necessary for contextual information processing, while the LS may be filtering contextual HPC information and relaying this signal to the LHb for proper adaptive behavioral output (Tsanov, 2018; Yetnikoff et al., 2015; Wirtshafter & Wilson, 2019).

Motivational State

The primary motivating factor of a living organism is the need for survival. Thus, an animal's experiences and adaptive actions in the world are highly influenced by recent previous experiences and motivations. For instance, an animal's willingness to work for food is influenced by whether or not they have eaten recently. There are many outstanding reviews on the neural circuitry underlying motivational systems (e.g. Berridge, 2004; Bromberg-Martin et al., 2010; Morales & Margolis, 2017; Petrovich, 2018; Burdakov & Peleg-Raibstein, 2020). Motivational systems research has typically focused on specific biologically-motivated behaviors such as hunger and feeding behaviors, as well as reproductive hormones and mate-seeking behaviors. The collective engagement of distinct and overlapping motivational systems define the specific profile of the current motivational state. Recent theories postulate that individual motivation structures not only process specific motivation information, but each structure is part of a collaborative effort to integrate motivation and cognition systems (e.g. Petrovich, 2018; Burdakov & Peleg–Raibstein, 2020). It is not yet known, however, how motivational states help to determine the specific type of response flexibility needed for accurate goal-directed and context-dependent navigation.

Motivational information is processed by the LHb

The LHb is known to receive a wide range of inputs relating to one's internal and external motivational state, such as value-based signals (Bianco & Wilson, 2009; Trusel et al., 2019), gustatory signals (Stamatakis et al., 2016), and circadian rhythm signals (Baño-Otálora & Piggins, 2017). For illustrative purposes, below we focus on only a few direct sources of motivation information that are known to modulate LHb activity, and possibly response flexibility.

The ENTOPEDUNCULAR NUCLEUS (EPN) provides significant motivational input to the LHb. Formerly thought to be primarily involved in motor movement (Hauber, 1998), LHb

lesions often resulted in cognitive and not motor-related deficits (Miller et al., 2006). Additional research unearthed another potential role for the EPN, implicating it in reward valuation (Hikosaka et al., 2006). Hong and colleagues (2011) found antidromic LHb signals in the globus pallidus (GP), a primate EPN analog, that differentially responds to reward. Bilateral inactivation of the GP led to the inability to learn new associations and task contingencies, implicating the GP in adaptive behavior (Piron et al., 2016). Interestingly, the EPN exhibits graded levels of firing activity corresponding to the expectation of an outcome, suggesting that the EPN encodes the value of an action as well as the outcome (Stephenson-Jones et al., 2016). These reward-related signals are sufficient to drive motivation. For example, Cerniauskas and colleagues (2019) found that most EPN neurons synapse onto VTA-projecting LHb neurons, driving LHb hyperexcitability and inducing motivational impairments. Thus, the EPN appears to communicate reward-related signals to the LHb, as well as contributes to driving motivation.

The LATERAL HYPOTHALAMUS (LH) also provides behaviorally-relevant motivational information to the LHb, regulating anxiety and depressive-like behaviors. The LH is functionally heterogeneous, containing both glutamatergic and GABAergic neurons, activation of which results in different behavioral responses (Jennings et al., 2013; Trusel et al., 2019). The LH has prominent projections that terminate in the LHb, and stimulating this projection results in both excitatory and inhibitory responses in the LHb, suggesting a potential bidirectional influence of LH on LHb activity (Stamatakis et al., 2016). In another study, orexinergic LH signals that terminate in the LHb result in LHb inhibitory responses, as well as increases in aggressive behavior (Flanigan et al., 2020). Excitatory responses in the LHb as a result of glutamatergic LH stimulation result in aversive behavior and it plays a role in generating a prediction signal for future negative events (Lazaridis et al., 2019; Lecca et al., 2017). As such, the LH exhibits a two-factor influence on LHb activity and subsequent motivated behavior.

The LATERAL PREOPTIC AREA (LPO) is a critical structure for motivational drive and it provides one of the largest inputs to the LHb (Yetnikoff et al., 2015). Comparable to other

structures that bidirectionally influence LHb activity, the LPO also exerts bivalent control over the LHb. Interestingly, glutamatergic and GABAergic LPO neurons simultaneously synapse on individual LHb neurons and both are activated by aversive stimuli (Barker et al., 2017). However, when stimulated individually, glutamatergic and GABAergic LPO inputs to the LHb produce divergent behavioral responses. This suggests that LPO activity is able to influence individual LHb neurons and drive opposing motivational states.

All mammals have a biological clock that regulates the activity of physiological functions (i.e. immune system coordination) and prepares them for specific motivated behaviors. The circadian rhythm is influenced by both intrinsic (i.e. physiological states, autonomic arousal) as well as extrinsic activity (i.e. daylight, food). For instance, animals that exhibit diurnal rhythms, like most primates, have increased motivation during the day to socialize and hunt for food. Without regular circadian rhythmicity, motivation lowers and animals tend to make less-optimal decisions (Acosta et al., 2020). Although many brain regions have been shown to play a role in this internal rhythmicity, the SUPRACHIASMATIC NUCLEUS (SCN) is the most prominent. The SCN organizes the activity in the brain that inevitably influences the body through its inherent ability to oscillate and synchronize the activity of multiple brain regions. The LHb appears to play an important role in circadian rhythmicity (Sakhi et al., 2014; Baño-Otálora & Piggins, 2017; Mendoza, 2017), likely as a result of receiving significant input from the SCN. Paul and colleagues (2011) found that LHb lesions resulted in motor and circadian rhythm impairment, implicating the LHb in the relay of SCN circadian rhythmicity important for behavior. It is possible that the LHb is integrating information about circadian rhythms to appropriately time motivated behaviors for optimal decisions (Mendoza, 2017). Lastly, reward-signaling structures (i.e. the VTA) have been shown to exhibit circadian rhythm firing, highlighting the LHb's circadian rhythm regulation of reward systems more generally (Bussi et al., 2014).

While the EPN, LH, LPO, and the SCN each strongly and directly relays motivational information to the LHb, it is worth noting that motivation information may bias the nature of information arriving in LHb from other structures not traditionally considered to be related to motivation, such as the mPFC. For example, in times of deliberation, an animal must evaluate options and select an action that would lead to the most optimal outcome. These actions most often have to do with approaching reward or avoidance of punishment. Selecting the action with the most optimal outcome involves evaluating similar previous actions and their respective outcomes. This manifests itself in the form of reward and reward prediction error signals (Bromberg-Martin & Hikosaka, 2011). Such value-based (motivation-related) signals are encoded at the time of action selection and outcome evaluation and are used to inform future behavior. The VTA and nucleus accumbens (NAcc) are likely involved early in this decision process since they seem to track outcomes and generate reward prediction signals as shown by neural activity that correlates with behavior and motivational effort for both the VTA (Bromberg-Martin et al., 2010) and NAcc (Hamid et al., 2015). NAcc activity, however, is not dependent on VTA input (Floresco et al., 1998). Instead, the basolateral amygdala (BLA), involved in reward-related associative learning, appears to drive NAcc reward firing. Importantly, these NAcc signals come to influence LHb activity which then drive downstream structures, such as the VTA, toward facilitation of reward approach or punishment avoidance behaviors (Bianco & Wilson, 2009). It is unclear whether the direct projection from the NAcc to the LHb influences motivated behavior.

The VENTRAL PALLIDUM (VP) receives significant reward-related signals from the NAcc and it in turn relays this information to the LHb via both glutamatergic and GABAergic projections (Soares-Cunha et al., 2020; Stephenson-Jones et al., 2020). Inhibition of excitatory VP inputs to the LHb abolished reward-seeking behavior, while inhibition of inhibitory VP inputs to the LHb abolished behavioral avoidance (Stephenson-Jones et al., 2020; Knowland et

al., 2017). Therefore, subpopulations of VP neurons (perhaps influenced by the NAcc) bi-directionally drive behavior via their inputs to the LHb in different motivational contexts.

The VTA-PFC projection has been implicated in many cognitive and behavioral processes such as mood regulation (Walsh & Han, 2014). Importantly, stimulation of VTA neurons induces neuroplastic strengthening of cortical inhibitory circuits, thereby inhibiting overall PFC activity (Zhong et al., 2020). Concurrently, dopamine injection increases theta coherence between the HPC and mPFC (Benchenane et al., 2010). It is possible that reward-related dopaminergic signals reach the PFC to update cortical information such that it more precisely represents the present situation relative to the HPC. In this way, information sent from the mPFC to the LHb is behaviorally relevant, thereby promoting timely and appropriate behaviors.

Motivational influence on contextual information

Behaviorally, many experiments have exemplified the positive influence of motivation, induced by factors such as reward magnitude, on performance in context-related goal-oriented tasks (Sänger & Wascher, 2011). Additionally, the presence of reward in specific predictable locations results in animals returning to these rewarded locations at a higher rate in the future (see Anselme, 2021 for review). It is possible that context information within PFC signals and motivational information from a number of subcortical structures arrive at LHb in a temporally precise manner that bias LHb outputs appropriate for a particular context. For example, Chang, et al. (1998) showed that a subset (~5%) of PFC and NAcc neurons respond to a rewarding stimulus simultaneously. At the population level, theta oscillatory synchrony between the PFC and VTA increases during actions that were likely to result in reward (Park & Moghaddam, 2017). The synchronized theta activity may be linking the PFC contextual information with the VTA reward signal, allowing the LHb to receive in a timely manner input to associate motivational and contextual PFC information.

The mPFC is thought to serve as an inhibitory control for motivated behaviors by interacting with the HPC to retrieve context-appropriate memories that inform future action selection (McDonald et al., 2008; Chen et al., 2013; Zelikowsky et al., 2013;). Interestingly, HPC neurons that project to the mPFC (Hsu et al., 2018), and mPFC neurons that project to the LHb (Mathis et al., 2021), appear to inhibit motivated behavior to seek reward, implicating this circuit in motivational regulation of reward seeking. The HPC itself has been shown to monitor and respond to motivational states when associated with a particular context (Kennedy and Shapiro 2009). Specifically, Kennedy and Shapiro (2009) show that HPC single units respond preferentially to a context paired with reward only when the rats were hungry or thirsty. This may be a result of motivational inputs directly influencing the HPC, as many of the aforementioned motivational circuits, such as the VTA (Gasbarri et al., 1994; Martig et al., 2009; Ghanbarian & Motamedi, 2013), SCN (Phan et al., 2011; McCauley et al., 2020), LH (Samerphob et al., 2015; Noble et al., 2019; Rezaee et al., 2020), EPN (Sabatino et al., 1986; Chen et al., 2020), and amygdala (Tsoory et al., 2007; Sheth et al., 2008; Ghosh et al., 2013), synapse onto and influence HPC activity. In sum, these findings demonstrated that the LHb is not alone in integrating motivational and memory information since cortical memory information likely already incorporates some aspects of motivation. What distinguishes the LHb from cortical systems that may be influenced by motivational and memory states is that LHb output may more directly determine optimal and adaptive behavior.

Motivation and memory-guided decisions

All previous experiences serve as roadmaps for future decisions, actions and their respective outcomes. The selection of an action that leads to a particular outcome will occur only when an animal is motivated. As mentioned above, the experience of hunger/satiation, the state of the biological clock, and internal valuation of possible outcomes define one's motivational states which guide and direct goals. As such, there is a necessary link between motivations and decisions, where highly motivated animals exhibit more effortful behavior in order to obtain a

reward. There are numerous studies examining the effect of motivational states on decision making, where animals must choose between small or large rewards that necessitate large or small amounts of effort, respectively (Floresco & Ghods-Sharifi, 2007; Mai et al., 2012). Animals will exert effort to seek reward up to a certain point, until the effort required is too great and no longer worth the payoff. This threshold is influenced by internal state and motivation, often changing depending on context (Knauss et al., 2020). Past experiences, too, shape internal state and motivation (Dysvik & Kuvaas, 2013). As animals evoke memories of similar previous experiences to evaluate the current context and most optimal choice, the animal's associations with a previous choice will influence their motivation and, subsequently, their decisions and actions. As a result of the functional interactions between the LHb and mPFC, the LHb serves as an integrative node for motivational and contextual information for the purpose of ensuring adaptive and flexible responses.

LHb and response or choice flexibility

Regardless of the species under study, it is often suggested that the habenula regulates an animal's ability to switch learned behavioral and cognitive strategies when a goal or context changes. This switch is likely not due to successive learning by different memory systems since strategy switching occurs much more quickly than new learning, and since multiple memory systems are thought to essentially operate in parallel (e.g. Mizumori et al., 2004; White et al., 2013; Hasson et al., 2015). The ability to rapidly change behavioral strategies is often attributed to the mPFC (e.g. Dalley et al., 2004; Ragozzino, 2007), but species without a defined prefrontal cortex (e.g. fish; Agetsuma et al., 2010; Okamoto et al., 2012; Stephenson-Jones et al., 2016) show remarkable abilities to flexibly respond in adaptive ways when a change in either the external sensory environment (including social cues, Chou et al., 2016) or internal state (such as motivation or use of learned task rules; Parker et al., 2012; Randlett et al., 2015, Cherng et al., 2020, Palumbo et al., 2020) occurs. Fish habenula, as an example, is often suggested to enable

response or behavioral flexibility by integrating the different types of information (including the evaluation of response outcomes, motivation state, and sensory cues) needed to strategically switch behavioral responses/strategies in simple and more cognitively demanding tasks.

The mammalian LHb (relative to the MHb) seems to have co-evolved with cortex to process more complex sensory and memory-related information, and this may enable more refined and flexible behavioral control when performing cognitive tasks that depend on limbic cortical processing (e.g. by HPC and mPFC; Ichijo et al., 2015, 2017; Mizumori & Baker, 2017). Early reports of the effects of lesions to the mammalian LHb showed that rats became unable to switch or maintain learned behaviors when contingencies changed in appetitive, HPC-dependent tasks (Thornton & Evans, 1984; Thornton & Davies, 1991). Importantly, LHb inactivation or lesion do not affect working memory, nonspecific sensory processing, identification of spatial locations, new learning, memory retrieval, motivation, behavioral activation, or reward discrimination per se (e.g. Baker et al., 2015, 2019, Lecourtier et al., 2004; Thornton & Evans 1984; Thornton & Davies 1991; Stopper & Floresco, 2014; Mathis et al., 2017; Mathis & Lecourtier, 2017). The hypothesis that the LHb importantly contributes to cortically-mediated response flexibility received additional support when rats were tested in HPC and mPFC-dependent tasks for which there is no right or wrong response, but rather choice preferences reveal an animal's responsiveness to changing task conditions. Such tasks require continuous and subjective value assessments to direct choice responses. LHb inactivation was found to be sufficient to disrupt such choice preferences when the probability of obtaining rewards shifted, or when the delay before reward access varied, during both operant testing (Shohamy et al., 2009; Dickerson et al., 2011; Delgado & Dickerson, 2012; Stopper & Floresco, 2014) and testing on open, elevated mazes (Baker et al., 2015, 2019). Importantly, spatial processing was not required for task performance, suggesting that the HPC involvement was related to other more integrative features of the task structure since changed preferences were observed only after the task structure shifted.

Understanding the specific processes and neural circuitry underlying the transformation between memory/motivational integration and the execution of behaviors is challenging since the LHb is not considered to be part of the motor output pathway that supports specific actions. Rather it is often explained that LHb's impact on response flexibility relies on its control over structures known to be important for the execution of voluntary actions (raphe nucleus and the VTA; e.g. Baker et al., 2019). Such an explanation is not satisfactory for it is still unclear how LHb-to-raphe or LHb-to-VTA signals can account for the type of response flexibility often attributed to the LHb. It is suggested here that one approach to resolving this apparent dilemma is to take a reverse engineering approach to this question. That is, the following starts by discussing the nature of the information represented by LHb neurons when rats are engaged in a HPC and mPFC-dependent natural foraging task. From there, we consider which of many brain structure(s) may be strategically informed by patterned LHb output to generate or enable flexible responses.

LHb neural representation during goal-oriented free navigation

If the LHb enables flexible behavioral responses to changing task conditions, one might expect LHb neural activity to somehow reflect this function. Indeed, the LHb has been shown to be a critical part of the neural circuit that generates prediction error signals when task conditions change. The well known dopamine neural response to prediction errors is driven at least in part by the LHb (Matsumoto & Hikosaka 2007; Bromberg-Martin and Hikosaka 2011; Proulx et al. 2014; Baker et al., 2015), even though this occurs indirectly through the rostromedial tegmentum, or RMTg; Li et al., 2019). While these findings illustrate that flexible behavior is likely mediated by more than one brain mechanism (e.g. Floresco, 2013), it clearly shows that LHb neural activity is driven by memory-based outcome expectations. Further evidence for the impact of experience on LHb neural responsiveness is the well documented change in LHb cell firing that occurs during aversive task performance as well as during stress

(e.g. Stamatakis & Stuber, 2012). Is the coding and integration of mnemonic and motivational information sufficient to ensure flexible responding? Recordings of LHb neural activity during a navigation-based foraging task shed new light on this question.

Using a pellet-chasing task that did not require HPC-based context memory, Sharp et al. (2006) described striking velocity-correlated neural activity in rat LHb. A subsequent study by Baker et al. (2015) confirmed the existence of prominent and strong (often $r > + 0.85$) velocity-correlated LHb neural activity but this time as rats performed a HPC-dependent spatial working memory task. This result was surprising given the general accepted view that the LHb contributes to learning and memory by signaling aversive/negative events/consequences/information (see review by Baker et al., 2016). However, Baker et al. (2015) also described another group of LHb neurons that responded to reward encounters, the expectation of rewards, and reward prediction errors in manners similar to the responses of primate LHb reward-responsive neurons described by Matsumoto and Hikosaka (2007). Further, about a third of the recorded LHb neurons showed conjunctive coding of reward and velocity information. During subsequent probe trials in which the reward condition or context was unexpectedly altered, the velocity correlate was retained albeit the overall firing rate was lowered. This pattern of reward and context coding by LHb neurons suggests that the LHb tracks the ongoing behavior of animals, but that the strength of movement state signals may be regulated by reward-related information. Perhaps this behavioral tracking feature is related to the recent report that LHb neurons encode a history of experiences (Andalman et al., 2019). A combination of reward and movement state neural signaling has also been reported for an important efferent structure of the LHb, the VTA (Puryear et al., 2010; Jo et al., 2013), and strong movement state information has been described for a major VTA afferent structure, the lateral dorsal tegmentum (LDTg; Redila et al., 2015). LDTg neurons were postulated to regulate reward responses of DA neurons according to the learned behaviors needed to obtain rewards. What might be the function of LHb movement state signals?

To aid in our understanding of the significance of LHb movement-related neural signals for response flexibility, it is helpful to first consider the finding by Aizawa et al. (2013) that spiking of LHb neurons are preferentially related in time to the peaks of simultaneously recorded HPC theta rhythms. While a strong argument was made that the LHb spike-HPC theta phase coherence resulted from a common input from the vertical limb of the diagonal band of Broca, this explanation does not address the issue of interest here, and that is how might LHb enable task-specific response flexibility. To shed new light on this issue, we offer the following hypothesis:

Hypothesis: During active navigation, the LHb may track and then relay information about one's ongoing behaviors to signal at appropriate times when downstream brainstem structures should drive hippocampal theta. In this way, LHb enables flexible responding not because it selects a particular action, but rather because it enhances a hippocampal neural state that is often associated with greater attention, arousal, and exploration. In freely navigating animals, these are essential conditions that are needed to discover and implement appropriate alternative choices and behaviors.

The HPC theta rhythm is known to encourage exploratory behaviors, increase arousal and attention, and improve learning and memory (e.g. Winson, 1978; Mizumori et al., 1989; Leutgeb & Mizumori, 1999; Lega et al., 2012; Buzsaki & Moser, 2013). Although the LHb does not have direct anatomical connections with the HPC, functional coupling between LHb and HPC has been demonstrated since LHb spikes exhibit cortical synchrony with HPC theta phase (Aizawa et al., 2013). Such spike-phase coherence is thought to reflect periodic influences of one structure on another (Singer, 1993; Engel et al., 2001; Buzsaki, 2002). Recent evidence (described below) suggests that a number of prominent LHb efferent targets in the hindbrain area may serve as important nodes for communication from the LHb and HPC. These structures receive substantial input from the LHb, and they are considered to be critical pacemakers for HPC theta. Thus, spiking activity in the LHb may enable response and cognitive flexibility by

regulating the HPC theta state to optimize exploration-related neural and memory plasticity. Such regulation could strengthen and/or maintain ongoing memory operations by the HPC during navigation of familiar contexts, as well as enable increased cognitive and response flexibility when task conditions change.

Transforming LHb neural signals into hippocampal neural states that support cognitive and response flexibility

The diverse array of LHb efferent targets is well documented (as reviewed in Baker et al., 2016; Kim, 2009; Mizumori & Baker, 2017). Of particular interest here are those that are considered theta-pacemaker structures for the HPC, such as the nucleus incertus, supramammillary nucleus, the median raphe, and the locus coeruleus (Figure 1.1). In contrast to the traditional view that hindbrain structures regulate slow processes such as the general state of arousal, recent findings demonstrate temporally and spatially-specific regulation of HPC physiology and behavior by these brain regions.

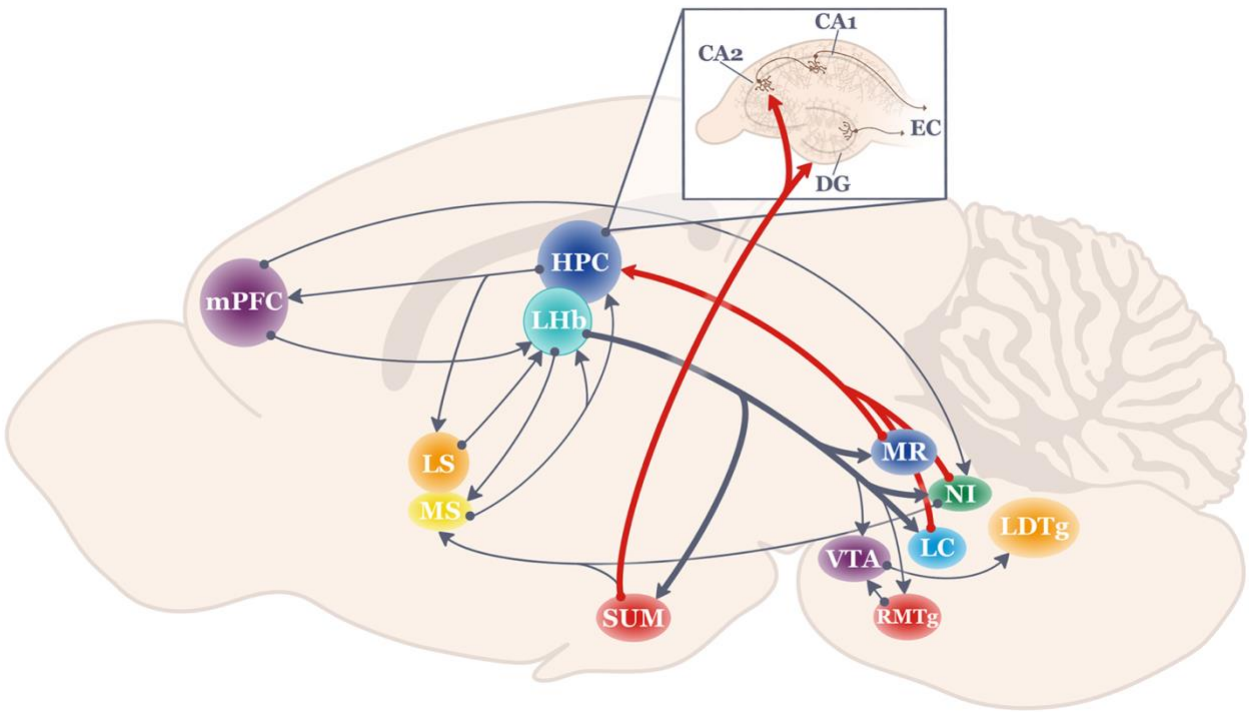


Figure 1.1

Schematic of the anatomical projections to and from the lateral habenula (LHb; shown in teal). Thick lines highlight LHb projections most strongly related to hippocampal function. HPC, hippocampus; LC, locus coeruleus; LDTg, lateral dorsal tegmentum; LHb, lateral habenula; LS, lateral septum; mPFC, medial prefrontal cortex; MR, medial raphe; MS, medial septum; NI, nucleus incertus; RMTg, rostromedial tegmentum; SUM, supramammillary nucleus; VTA, ventral tegmental area.

The NUCLEUS INCERTUS (NI) lies in the midline periventricular central gray region of the pontine hindbrain, and it is generally considered to be an essential part of the ascending reticular activating system (Steriade & Glenn, 1982) since it innervates structures known to regulate HPC theta such as the MS (Vanderwolf, 1969; Vertes & Kocsis, 1997; Goto et al., 2001; Olucha-Bordonau et al., 2003; Ma et al., 2013). Also, stimulation or inhibition of the NI up- or down-regulates active locomotion (respectively), as well as modulates physiological indices of arousal and HPC theta (Nunez et al., 2006; Lu et al., 2020). For example, NI neurons preferentially fire at the initial ascending phase of the HPC theta rhythm (Ma et al., 2013), possibly to reset theta (Martinez-Bellver et al., 2017). Evidence that NI impacts on HPC physiology have consequences for HPC-based memory has been demonstrated using a variety of behavioral tasks (Gil-Miravet et al., 2021), including context fear conditioning (Szonyi et al., 2019), various maze-based tasks (Nategh et al., 2015; Albert-Gasco et al., 2017), as well as spatial working memory operant tasks (Albert-Gasco et al., 2017; Garcia-Diaz et al. 2019).

GABA and glutamate incertus neurons (Lein et al., 2007; Cervera-Ferri et al., 2012) express receptors for stress-related hormones (corticotropin-releasing factor, or CRF) and contain multiple neuropeptide markers for stress and arousal such neuromedin, relaxin, D2 dopamine receptors, and orexin/hypocretin (e.g. Jennes et al., 1982; Kubota et al., 1983; Ma et al., 2013; Lu et al., 2020). Therefore, the NI had been studied primarily for its role in different physiological states. However, as noted above, more recent emerging evidence clearly shows that especially the relaxin-3 NI neurons likely play a significant role in HPC -based memory (Gil-Miravet et al., 2021). For example, these relaxin-3 neurons are the ones that preferentially fire at the initial ascending phase of the HPC theta rhythm (Ma et al., 2013). Furthermore Szonyi et al. (2019) identified an incertus- HPC circuit that may determine which CA1 pyramidal neurons take part in context memory processing: NI long-range GABAergic neurons project directly and indirectly (via the medial septum) to HPC somatostatin neurons to regulate the excitatory/inhibitory balance in stratum oriens of CA1, thereby helping to select which cells

participate in memory networks and which ones do not. Since the NI receives strong projections from the prefrontal cortex and the LHb (Goto et al., 2001; Lu et al., 2020), one or both of these incertus afferent systems may drive the NI to regulate HPC neural activity in context-specific ways.

The SUPRAMAMMILLARY NUCLEUS (SUM) is another deep brain structure of interest when considering how LHb neural activity may translate to HPC-mediated response flexibility. The SUM is a hypothalamic structure that provides strong direct and indirect (via the medial septum) theta-rhythmic inputs to the HPC (Haglund et al., 1984; Vertes, 1992; Kirk & McNaughton, 1993; McNaughton et al., 1995; Vertes & Kocsis, 1997; Vertes & McKenna, 2000; Vertes, 2015; Ito et al., 2018). The overall functional impact of the SUM input is to not only generate HPC theta (Pan & McNaughton, 2004), but SUM afferents amplify neocortical input to the HPC by increasing the responsiveness of dentate gyrus granule cells to input from the entorhinal cortex through disinhibition of inhibitory interneurons (Mizumori et al., 1989a). Such regulation of the excitatory state of granule cells may selectively promote information arriving from the entorhinal cortex. More recently, behaviorally-relevant functional coupling between the SUM and the HPC was shown when SUM spiking became HPC theta phase-modulated particularly before choice points in a continuous alternation task (Ito et al., 2018). Specifically, SUM cells became aligned to the later phases of the CA1 theta cycle, and SUM spiking began close to the time of firing by CA1 interneurons. In the same study, the SUM was demonstrated to be a critical coordinator of communication within the HPC-mPFC-nu. reuniens memory circuit. Thus, it was suggested that the SUM (and by extension, the LHb) dynamically coordinates HPC-related memory circuits by varying spiking relative to HPC theta (Ito et al., 2018).

Not only does the SUM regulate intra- and extra-HPC information processing, but recently it was elegantly demonstrated that the SUM may provide and/or facilitate specific types of information processing in the HPC via its distinct direct inputs to the dentate gyrus and

region CA2 (Vertes & McKenna, 2000). The SUM to dentate gyrus input may signal contextual novelty while the SUM to CA2 input may signal social novelty (Chen et al., 2020). Thus, the SUM appears to be not only involved in the generation of the HPC theta, but it is also important for gating contextual and social information processing within the HPC. Further, SUM enables HPC to communicate with partnered mnemonic structures such as the mPFC and the nu. reuniens. Given the multiple ways that the SUM impacts HPC processing, it is not surprising that lesion or reversible inactivation of the SUM has been shown to impair different types of HPC -dependent behaviors as shown on spatial working memory and certain avoidance tasks (Shahidi et al., 2004; Aranda et al., 2006, 2008), and to be activated during times of stress (Wirtshafter et al., 1998; Ito et al., 2009; Choi et al., 2012). Given the strong input from the LHb to the SUM (Kiss et al. 2002), the SUM is a strong candidate for linking functions of the LHb and HPC (as noted by Goutagny et al., 2013). Here we hypothesize more specifically that the theta-rhythmic firing of SUM neurons may be regulated by LHb behavioral/movement state signals, and in this way LHb output can generate the conditions needed for animals to flexibly respond to changes in task conditions.

The MEDIAN RAPHE (MR) has long been studied for its role in emotion and stress regulation (e.g. Andrade et al., 2013; Graeff et al., 1996). Recently (Baker et al., 2015), it was suggested that the MR may also play a critical role in the coordination of communication between the LHb and HPC since the MR receives strong input from the LHb (Quina et al., 2015; Metzger et al., 2017), the MR has strong projections to a broad extent of the HPC (Azmitia & Segal, 1978; Vertes et al., 1999) and the MR has been shown to significantly impact HPC-dependent behaviors and theta/ripple oscillations (Vertes & Kocsis, 1997; Wang et al., 2015) Thus, the MR is strategically situated to at least assist in the transformation of LHb signals to regulate HPC theta in a manner that facilitates response flexibility. Indeed, a link between serotonergic function and response flexibility is often discussed given the numerous studies that report alterations of serotonin receptors or neurotransmitter release results in difficulties

performing classic response flexibility tasks such as set shifting and strategy shifting (excellent reviews include Nilsson et al., 2015; Alvarez et al., 2021).

The LOCUS COERULEUS (LC) is another hindbrain structure that (in anesthetized and awake rats) is known to project to HPC to impact theta and gamma oscillations (Gray et al., 1975; Walling et al., 2011; Broncel et al., 2021), and to receive at least modest input from the LHb (Mathis et al., 2021). As reviewed by Sara (2009) and Poe et al. (2020), LC neural firing has long been observed to occur in response to novelty, to signal-mismatches events, and to attentional shifts (Aston-Jones & Bloom, 1981, Herve-Minvielle & Sara, 1995; Aston-Jones et al., 1991; Sara & Segal, 1991; Bouret & Sara, 2005). Our cumulative understanding of the broad impact of the LC across many brain regions as well as the diverse cell types and patterns of LC neural activity have led to theories that especially phasic LC activity enhances the execution of actions in response to unexpected stimuli (Aston-Jones & Cohen, 2005). LC activation, then, effectively resets neural networks to enable rapid switches of cognitive representations or response strategies when needed (Sara & Bouret, 2012). These types of LC responses appear necessary for animals to exhibit adaptive behaviors (Yu & Dayan, 2005). Thus, in addition to the NI, SUM, and MR, the LC is a strong region of interest for its putative role in transmitting LHb signals to the HPC by establishing a HPC theta state that supports response and cognitive flexibility (Figure 1.2).

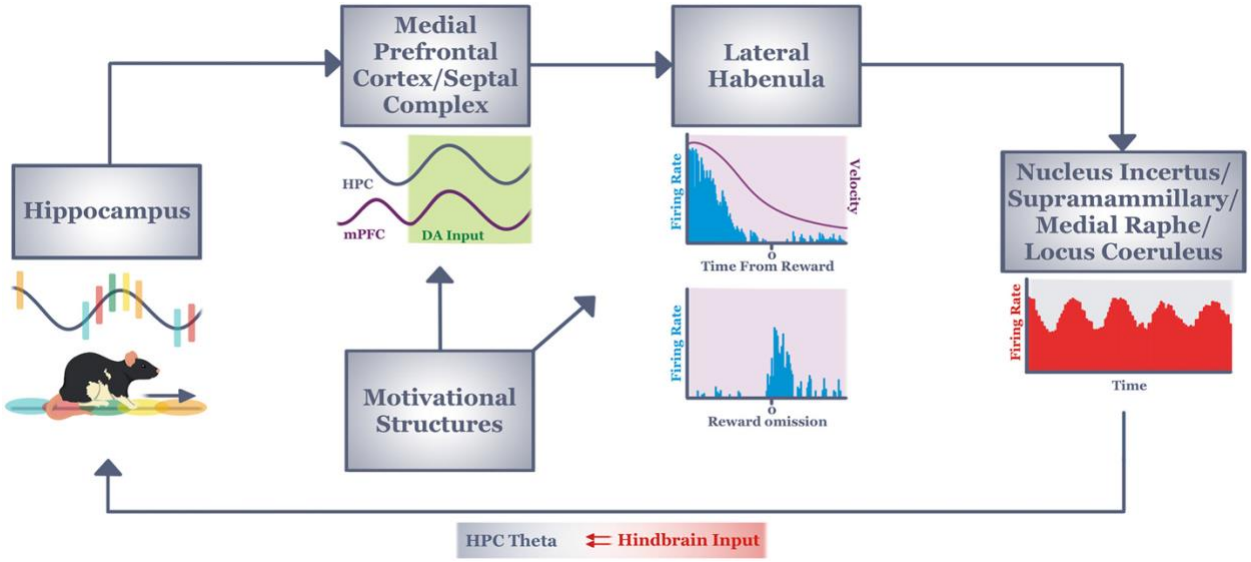


Figure 1.2

Information flow through our hypothesized circuit (see text) that mediates context-dependent response flexibility during active navigation. Context information from the hippocampus (e.g. in the form of place field sequences) informs decision processes of the medial prefrontal cortex and septal complex via reward-modulated theta coherence. The output of this memory/decision system integrates with motivational information in the lateral habenula. Lateral habenula neurons integrate this information over many seconds to discharge tonically (to encode current behavioral state, or velocity) or phasically (to encode short duration stimuli/events such as reward outcome). Both types of signals are driven by motivational, memory and decision processing (see text). The output of lateral habenula is hypothesized to provide a signal to hindbrain regions known to regulate hippocampal theta. In this way, the lateral habenula may establish/maintain a neural state in the hippocampus that encourages the increased attention and exploratory tendencies needed for animals to respond flexibly to changing task conditions. DA, dopamine; HPC, hippocampus; mPFC, medial prefrontal cortex.

LHb contributes to response flexibility along multiple timescales: implications for understanding LHb-related psychopathologies

Multiple time scales of LHb neural responses and the impact on response flexibility

Based on our extant knowledge and theories about how the LHb contributes to response flexibility, an important and fundamental question arises: How does one reconcile findings that the LHb signals short duration aversive events (Matsumoto & Hikosaka, 2007), while also signaling longer-duration behavioral/movement states (Baker et al., 2015). One explanation is that while LHb broadly tracks the current sensory/behavioral situation, LHb neurons exhibit different patterns of firing depending on the types of information being tracked. LHb neurons may fire phasically to single, short-duration events such as sensory stimuli or a particular goal outcome, or they may fire tonically during longer-duration behavioral state conditions such as movement through space. This sort of dual-coding is not uncommon in the brain. One example is that the same VTA dopamine neurons fire phasically to rewards, and tonically when moving through space (e.g. Puryear et al., 2010; Jo et al., 2013). Either short- or long-duration firing could impact response flexibility that is enabled by the LHb efferent structures described earlier. For example, encountering an unexpected reward should result in phasic LHb cell firing, which would signal efferent structures to increase HPC theta to resolve a potential conflict between expected and actual context features (Mizumori et al., 1999). When tracking sensory/behavioral information across this relatively short period, the LHb may be serving as a brake signal to halt initiation of potential inappropriate behaviors thereby allowing other adaptive behaviors to commence (Sleezer et al., 2021). When faced with other aversive situations, LHb output may instead result in the engagement of avoidance behaviors. In either case, LHb activity is considered critical during flexible action selection.

By comparison, tonic LHb neural activity for example during spatially-extended navigation, should signal hindbrain regions to elevate HPC theta for the duration of the

translocation in space. Such signaling is postulated (see above) to enable the timely enhancement of neuroplasticity mechanisms needed to evaluate the current context so that memories can be updated in preparation for a future decision. That is, LHb-induced HPC theta may result in greater attention and arousal, along with a stronger neuroplasticity state that provides the basis of more efficient and timely context evaluation. Such context analysis is considered essential in order to then execute adaptive and flexible responses in the future. During real world navigation, the LHb engages in both short (sensory) and intermediate term (during navigation) behavioral monitoring in the same context as evidenced by LHb neurons that show both reward and velocity-related neural codes (Baker et al., 2015). Such dual time frame coding may not be needed in Pavlovian or operant tasks that do not require animals to move about in a spatially-extended environment, but rather they only require information to be associated over relatively short time scales. Thus, assessing LHb in navigating animals may reveal an extended complement of potential contributions to adaptive responding.

In sum, we propose a) that the LHb contributes to response flexibility by tracking behavior across short (in the tens of milliseconds to seconds range) and intermediate (in the range of seconds to hours or more) time frames. Prolonged disturbances of function in either of these time domains can result in different behavioral disorders and psychopathologies.

LHb dysfunction in the short and intermediate term

LHb responses to short-term aversive or appetitive stimuli are necessary for proper adaptive behavior and does not typically result in behavioral maladaptations (Baker et al., 2015). As shown in many of the experiments described previously, inaccurate stimulus coding, however, results in suboptimal decisions. If LHb encoding of behavioral and movement states are deficient, one would expect impaired and inappropriate activation of HPC theta, which could lead to memory deficits especially with flexible responding is needed. We also know, based on extensive research on the role of LHb in processing aversive information, the LHb plays a

substantial role in fear memories. For instance, in the case of fear conditioning, it is important to learn the association between a specific context and the potential threat. In aversive situations, not only does the LHb respond to the aversive stimulus, but also to the cue predicting the onset of the aversive stimulus (Trusel et al., 2019; Lazaridis et al., 2019; Lecca et al., 2017). It takes a mere 5 trials in order for short-scale synaptic changes to occur in the LHb, likely between the LHb and the RMTg (Wang et al., 2017). Also, inactivation of the LHb following fear conditioning of a context resulted in context memory impairments (Durieux et al., 2020). Furthermore, in the same experiment, c-fos expression following fear conditioning increased in the HPC, mPFC, as well as the LHb, suggesting an involvement of this circuit in contextual fear memories. Thus, in situations when the LHb does not appropriately function over periods of seconds to minutes to hours, one sees impaired decision making and poor context memory.

Prolonged LHb dysfunction

Extended and hyperactive LHb aversive signaling can lead to long lasting plasticity-related changes, resulting in psychiatric disorders such as depression, schizophrenia, and addiction. In support of this theory, studies have shown that cocaine exposure induces increased AMPA receptor expression in excitatory LHb neurons, causing long-term potentiation and hyperexcitability (Maroteaux & Mameli, 2012). Similarly, prolonged exposure to an aversive stimulus, such as a foot shock, results in decreased GABAb receptor expression in excitatory LHb neurons, leading to disinhibition and hyperexcitability of LHb neurons (Lecca et al., 2016). Along with synaptic changes, stress and other environmental factors can induce changes in LHb gene expression (Levinstein et al., 2020). Specifically, expression of genes implicated in the RMTg, and not VTA or DRN, pathway is increased in the LHb following stress. These genetic changes alter the strength of the connections between the LHb and downstream structures, resulting in long-lasting changes in plasticity.

Excessive LHb activity can also lead to impaired motivated behavior and result in the pathophysiology of depression. In fact, the LHb is the only brain region that exhibits consistent hyperactivity in depression (Caldecott-Hazard et al., 1988; Andalman et al., 2019). One of the pathways implicated in the expression of depression-like symptoms is the LHb-RMTg pathway that inhibits dopaminergic activity in the VTA and other structures (Proulx et al., 2018). Another pathway underlying this disorder is the LHb's increased excitation of raphe inhibitory interneuron-based inhibition of serotonergic neurons, which causes a passive coping transition, a marker of depression (Andalman et al., 2019). Ketamine, a common antidepressant medication, has shown to elevate raphe activity in addition to decreasing the hyperactivity of the LHb, consistent with the role of this pathway in depression (Lopez-Gil et al., 2019; Yang et al., 2018; Cui et al., 2018).

Maladaptive activity in the PFC is frequently tied to the phenotype of schizophrenia (Weinberger et al., 2001). Specifically, the established dopamine hypothesis of schizophrenia posits that schizophrenia arises as a result of dopaminergic hyperactivity in subcortical areas due to cortical dysfunction (Winterer & Weinberger, 2004). In a rat model of schizophrenia, Li and colleagues (2019) found that there was significant hypofunctionality in the LHb, potentially disinhibiting subcortical dopamine activity. In the same experiment, lesioning the LHb of schizophrenic rats resulted in a significant decrease in cortical activity. Interestingly, functional connectivity between the habenula and cortex increases in schizophrenic patients, suggesting that the habenula may be contributing to the cortical dysfunction seen in schizophrenia (Zhang et al., 2017). Moreover, serotonergic activity in the DRN increases in the pathophysiological profile of schizophrenia. Typically, in healthy brains, the LHb functions to inhibit serotonergic activity in the DRN. However, in schizophrenia, LHb activity is hypoactive, disinhibiting serotonergic activity in the DRN. When the LHb is lesioned in schizophrenic rats, serotonin levels in the DRN, as well as in the mPFC, is increased (Li et al., 2019). Owing to these findings, hypofunction in the LHb may contribute to the pathophysiology of schizophrenia.

Likely as a consequence of its critical role in reward valuation and processing, the LHb plays a large role in addiction. Repeated drug exposure results in addiction, or excessive drug seeking behavior, and is tied to increases in LHb activity (Zhang et al., 2005). Although the LHb processes both aversive and rewarding properties of stimuli (Matsumoto & Hikosaka, 2009), it appears that the LHb primarily responds to the aversive properties of drug exposure (Zhang et al., 2013). In particular, LHb activity is thought to underlie the negative effects of drug seeking behavior through its projection to the RMTg (Meye et al., 2015). In support of this finding, Maroteaux and Mameli (2012) showed that cocaine exposure results in increased AMPA receptor expression in LHb neurons selectively projecting to the RMTg. Lastly, chronic drug exposure causes neurodegeneration in the main output fiber bundle of the LHb, the fasciculus retroflexus, disrupting the LHb's communication with and modulation of downstream monoaminergic structures (Lax et al., 2013).

Therapeutic treatment for LHb dysfunction

Reasoning that long term LHb function underlies maladaptive behavior, clinicians target the LHb in an attempt to correct LHb dysfunction as a therapeutic approach. Deep brain stimulation (DBS) of the LHb has been one such therapeutic procedure that is producing encouraging results (Sartorius et al., 2010). Interestingly, DBS frequency parameters exhibit differential therapeutic outcomes in distinct disorders such as depression and addiction. For an in-depth discussion of DBS in the LHb, see the excellent review by Germann and colleagues (2021). Relatedly, ketamine has proven to be a successful therapeutic treatment that inhibits the LHb and disinhibits reward centers such as dopamine and serotonin (Yang et al., 2018).

Another emerging therapeutic approach for the development of treatments for LHb-related psychopathology focuses on the serotonergic system. Evidence suggests that serotonergic dysfunction results in the inability to adapt to changing environmental conditions, as seen in depression and addiction. Interestingly, the effects of serotonin depletion or

overexpression do not always resemble one another across studies. For instance, Lapiz-Bluhm (2009) showed that chronically stressed rats that were serotonin-depleted using para-chlorophenylalanine showed significant deficits in reversal learning which was rescued with a serotonin reuptake inhibitor. In a separate study, humans were exposed to a rapid tryptophan depletion paradigm on a reversal learning task and exhibited slightly improved decision-making (Talbot et al., 2006). Such discrepancy in the literature may be attributed to inter-species differences, as well as the reliability of the drug cocktail protocol. Recent evidence has exposed distinct functions of serotonin subtypes as the main culprit (Alvarez et al., 2021). Importantly, Morris and colleagues (1999) showed that selective serotonin depletion causes significant increases in LHb activity, mimicking the neural correlate of depression, compared to other structures. Furthermore, tryptophan depletion causes a significant impairment in context memory in mice, implicating the serotonergic system in both motivation and context memory (Uchida et al., 2007). Serotonin 2A receptors subtype has been extensively studied in relation to its influence on behavioral flexibility. A selective serotonin 2A receptor blockade significantly impaired performance on a spatial reversal learning task (Boulougouris et al., 2008). In the same study, a selective serotonin 2C receptor blockade improved performance on a spatial reversal learning task. Interestingly, serotonin 2C and serotonin 2A receptors are found on GABAergic dorsal raphe neurons (Serrats et al., 2005). Psilocybin, being studied as an antidepressant, is a strong serotonin 2A receptor agonist and serotonin 2A receptors are highly expressed in the LHb. This raises the question as to whether psilocybin has modulatory effects on LHb activity. Indeed, activation of serotonin 2A receptors inhibits excitatory LHb neurons, likely through the facilitation of GABAergic transmission, suggesting that psilocybin mimics a neuromodulator and work to rescue LHb dysfunction (Fig. 3; Shabel et al., 2012; Metzger, et al., 2017). In support of this, psilocybin administration has proven effective in drug-resistant depression (Carhart-Harris et al., 2018), and addiction (Johnson et al., 2014). Whether the

therapeutic effects of psilocybin result from the direct action on LHb activity, however, is not yet clear and is in need of further study.

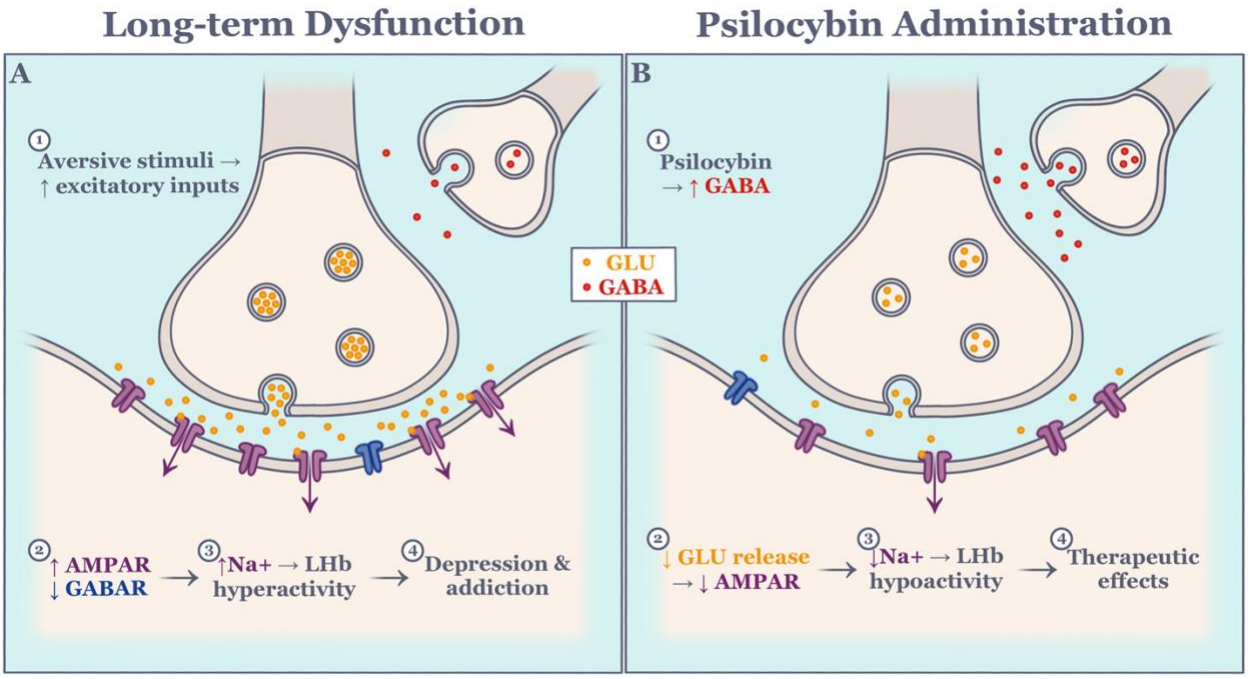


Figure 1.3

Schematic of hypothesized molecular effect on excitatory lateral habenula neurons during long-term dysfunction (A) and psilocybin administration (B). Events are numbered in succession. AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; GABA, gamma-aminobutyric acid; GABAR, gamma-aminobutyric acid receptor; GLU, glutamate; LHb, lateral habenula; Na, sodium.

The diversity of LHb inputs from cortical and subcortical areas makes the LHb a prime region of integrating information related to context memory and motivation. Likewise, the LHb's downstream control of monoaminergic centers, as well as the HPC, implicates it in a number of psychiatric disorders, such as those described above. The reviewed data provide a compelling argument that the LHb should be one of the primary targets of therapeutic intervention, such as with psilocybin, for psychiatric disorders that manifest in context memory, motivational impairments, and certain disorders of behavioral control.

Conclusion

An outstanding and challenging question is how the LHb enables response flexibility. Here, it is hypothesized that LHb may enable response flexibility by integrating context memory (e.g. from HPC and mPFC) and internal state information to provide critical feedback to memory systems (e.g. the hippocampus) about the outcome of choices and the status of behaviors (e.g. movement velocity). Importantly, this feedback may upregulate neural states in HPC when a context change requires flexible responding to maintain accurate decisions. The upregulation of at least HPC theta could enable the greater attention, arousal, and behavioral activation needed for response flexibility. This feedback system to the HPC, then, may represent a critical step in the loop of information processing between context memory and decision systems, intrinsic motivational systems, response implementation, and memory updating and retrieval that is needed to flexibly redirect responses. Supporting our hypothesis, improper functioning of the LHb results in impairments in behavior related to response flexibility such as those seen in psychiatric disorders.

To directly assess the contribution of the HPC-mPFC-LHb circuit to flexible responding and adaptive behavior, we inactivated the LHb with muscimol and tested rats on a spatial set-shifting task as we monitored trial-to-trial flexibility and strategy use. We then approached the circuit from the other direction by inducing a maladaptive state of morphine withdrawal and

examining the effects on flexible behavior on the same task. To probe the extent to which contextual processing occurs in the mPFC, we also recorded calcium activity from the mPFC on a hippocampal-dependent novel object recognition task as rats were undergoing morphine withdrawal. Lastly, to examine the role of the serotonergic system during morphine withdrawal, we tested the therapeutic effects of psilocybin both at the neural level as well as its efficacy in reversing the morphine-induced flexibility-related impairments.

Chapter 2

The lateral habenula as a link between negative outcomes and adaptive strategy switching

Hones et al., 2026.

Introduction

Behavioral flexibility and adaptation

The ability to quickly and flexibly respond in a dynamic world is paramount for survival. Animals use complex strategies to optimize responses necessary for vital behaviors, such as foraging, while managing the trade-off between predation risk and energy expenditure (Ladds et al., 2020). As such, there is an evolutionary advantage to form an accurate representation of the environment to respond flexibly and appropriately to changes that might arise. This form of behavioral adaptation is often referred to as 'behavioral flexibility' (Poirier 1969). Although behavioral flexibility is frequently beneficial, it does not always result in an optimal outcome (Lea et al., 2020). Therefore, it is useful to consider that behavioral adaptation also incorporates the concept of 'behavioral stability,' which refers to the stability of responses despite changes in the external environment (Serrien et al., 2019; Uddin et al., 2021). For instance, returning to the same foraging site is a relatively inflexible behavior but may be advantageous when resources are consistently available. Conversely, if recent choices have led to suboptimal outcomes (i.e. lack of resources or dangerous situations), animals need to adopt alternative behavioral strategies to optimize results and decrease risk. The appropriate balance of flexible and stable responses, then, reflect adaptive decision making towards goal optimization.

The lateral habenula and its role in behavioral flexibility

The lateral habenula (LHb) is an evolutionarily conserved epithalamic structure that is present in all vertebrate species (Grillner et al., 2018). Historically, the LHb has been known to

play a role in aversive and negative outcome experiences (Gao et al. 1996; Matsumoto & Hikosaka, 2007), in part due to its unique anatomical connections with monoaminergic structures that receive signals about aversive and/or negative events. Specifically, the LHb has both direct and indirect influences over the dopaminergic ventral tegmental area (VTA; Bernard & Veh, 2012) and serotonergic median and dorsal raphe nuclei (MRN and DRN, respectively; Wang & Aghajanian, 1977). Influence over monoaminergic structures also allows the LHb to guide behaviors related to reward and motivation (Lammel et al., 2014; Li et al., 2016) as well as the ability to flexibly cope with changing task contingencies (Coffey et al., 2020). It appears that the LHb's interaction with downstream midbrain structures (VTA, MRN, DRN) likely plays a role in updating choice value for appropriate and flexible responding (Baker et al., 2015; Baker & Mizumori, 2017; Baker et al., 2019; Palumbo et al., 2020).

The LHb's precise contribution to behavioral flexibility is likely complex since the ability to engage behaviors flexibly requires multiple active processes, e.g. disengaging from prior behaviors, exploring alternative strategies, selecting the most optimal approach, and executing an action. Past studies investigating the role of the LHb in flexible responding have employed reversal learning (Baker et al., 2015), delay discounting (Shohamy et al., 2009; Dickerson et al., 2011; Delgado & Dickerson, 2012; Stopper & Floresco, 2014), and spatial delayed-alternation tasks (Baker et al., 2019). These tasks rely on error and trials-to-completion metrics to determine that LHb contributes to flexible responding. The precise reasons that the LHb contributes to response flexibility, however, remains unclear. That is, it is not understood whether the LHb contributes to response flexibility by enabling the disengagement from previous strategies, the ability to engage with new strategies, or the deliberation of potential choice outcomes.

The present study utilized a spatial set-shifting task (Miles et al., 2024) to assess, on a trial-by-trial basis, the specific contributions of the LHb to adaptive behavior. Inactivation of the LHb resulted in impaired strategy adaptation when reward contingencies changed, and

dampened the ability to utilize incorrect choice outcomes towards appropriate subsequent strategy adaptation. Additionally, LHb inactivation disrupted the typical surge in deliberative behavior prior to the change in reward contingencies, as well as prior to the point at which the new strategy is learned. The results of this study provide new insights into how the LHb might contribute to the evaluation of negative choice outcomes to enable flexible and accurate responses.

Results

The spatial set-shifting task was conducted on a fully-automated, elevated plus maze. Two opposing maze arms (North and South) served as randomly assigned trial start locations, and the ends of the other two opposing arms (East and West) served as potential reward locations. Both reward locations were initially accessible on all choices, but the rewarded location changed throughout the task based on target strategy (see Materials & Methods - Apparatus & Training Procedures for full details). Training took an average of ~19 days (ranging from 13-28 days) to complete. Rats took an average of 3 days to reach asymptotic performance following surgery. To test whether the LHb contributes to adaptive changes in strategy use, muscimol, a GABAa agonist, was infused locally into the LHb in an ABBA format to temporarily inactivate the structure as rats performed a complex spatial set-shifting task (Figure 2.1A, B). Basic performance on the set-shifting task was compared in muscimol-infused sessions to saline-infused control sessions. To examine whether LHb inactivation had an effect on locomotor behavior, the time it took for rats to complete a given trial within a session was measured. The average time to complete a trial was ~16 seconds, with no significant differences between conditions (Figure 2.2A, $t(7)=-1.512$, $p=0.174$). To confirm that the effects of muscimol were temporary and did not last beyond 2 hours after infusion (Allen et al., 2008), differences in block completion and overall choice performance was compared between the first and second infusions of muscimol (see Methods), which were 24-48 hours apart. The number of blocks and

percent of correct trials that rats were able to complete between the first and second infusions remained the same (Supplementary Figure 2.1A, B, C). As a result, the rats' performance across the 2 A and the 2 B sessions were averaged for all analyses.

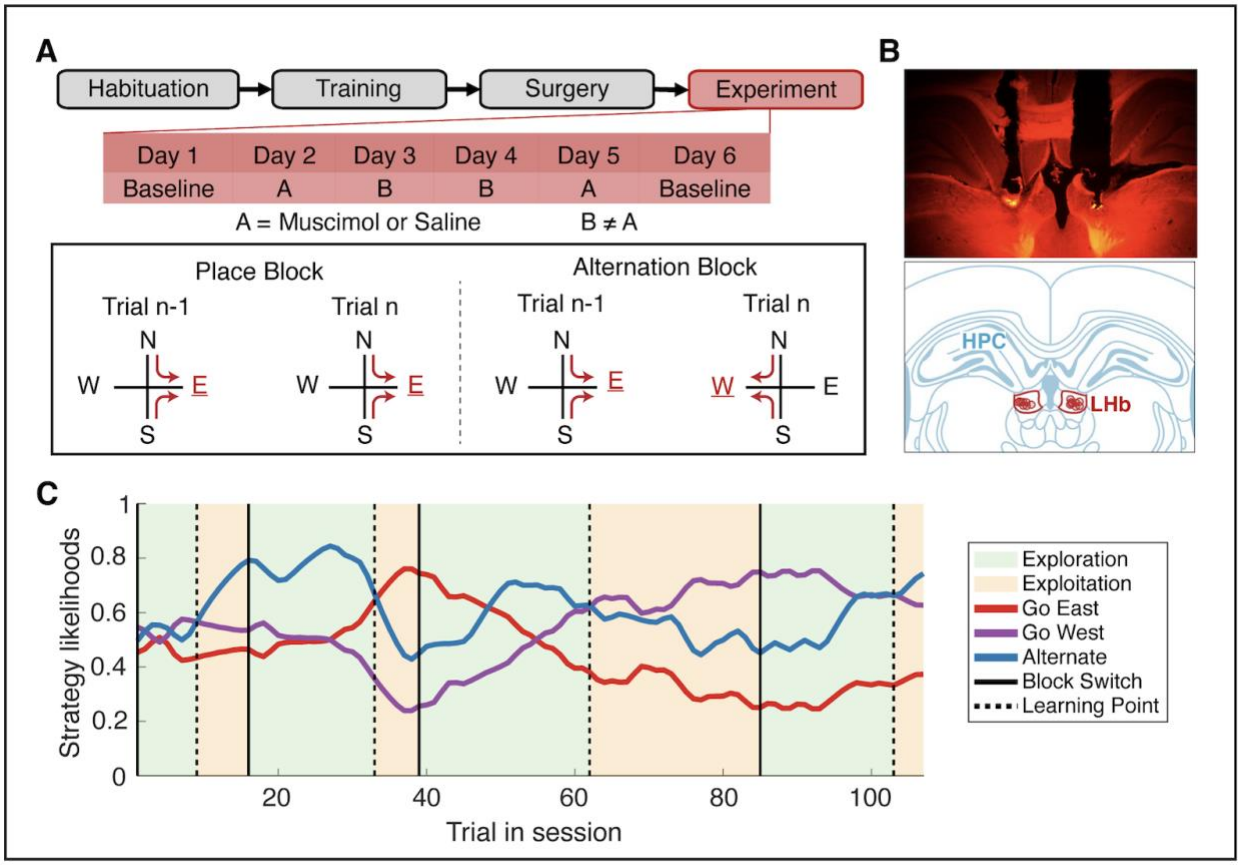


Figure 2.1

Experimental paradigm and histological results. A schematic representation of the sequential training steps and experimental paradigm (A, top) and diagram of task rules (A, bottom). Histological example of bilateral cannula placement marked by DiI in the LHb (B, top) and schematic of bilateral cannula placement in eight rats indicated by red circles (B, bottom). An example session estimating the strategy likelihood curves superimposed on the trials in the session, along with key task points and phases (C).

LHb Inactivation Delays Strategy Adaptation

To directly assess the effect of the LHb infusions, behavioral responses to muscimol were compared to those of saline. The total number of blocks that rats completed within a single session was analyzed. LHb inactivation significantly decreased the number of blocks rats completed in a session compared to saline (Figure 2.2B; $t(7)=2.393$, $p=0.048$). For subsequent analyses, trials from incomplete blocks were removed to determine whether LHb inactivation affected choice behavior during completed blocks on the task (e.g., if an animal completed three blocks and did not complete the fourth, only data from the first three completed blocks were analyzed). Results showed that LHb inactivation significantly decreased overall choice accuracy on the task (Figure 2.2C; $t(7)=2.708$, $p=0.030$). Additionally, LHb inactivation significantly increased the number of trials it took rats to complete a block (Figure 2.2D; $t(7)=-4.567$, $p=0.003$). When examining the number of trials it took rats to complete a block by individual block, muscimol did not significantly affect trials per block. However, although these effects did not reach significance, later blocks in the session trended towards greater impairment (Figure 2.2E; block 1: $t(46) = -0.232$, $p = 0.817$; block 2: $t(46) = -1.531$, $p = 0.265$; block 3: $t(46) = -2.296$, $p = 0.079$; block 4: $t(47.3) = -2.535$, $p = 0.058$).

To understand whether LHb inactivation impaired switching from a previously learned strategy to a new one, we estimated the number of trials it took to reach the learning point, which was defined as the exploration period. This differs from the number of trials it took to complete a block after learning the target strategy, which was defined as the exploitation period. LHb inactivation significantly increased the number of trials it took to explore potential strategy options (Figure 2.2F; $t(7)=-2.514$, $p=0.040$), but did not affect the number of trials during exploitation (Figure 2.2G; $t(7)=-1.175$, $p=0.278$) relative to saline control sessions. These results suggest that LHb inactivation impaired rats' ability to flexibly adapt to a new strategy following a block switch. However, once the target strategy was adopted on a given block, it was successfully exploited.

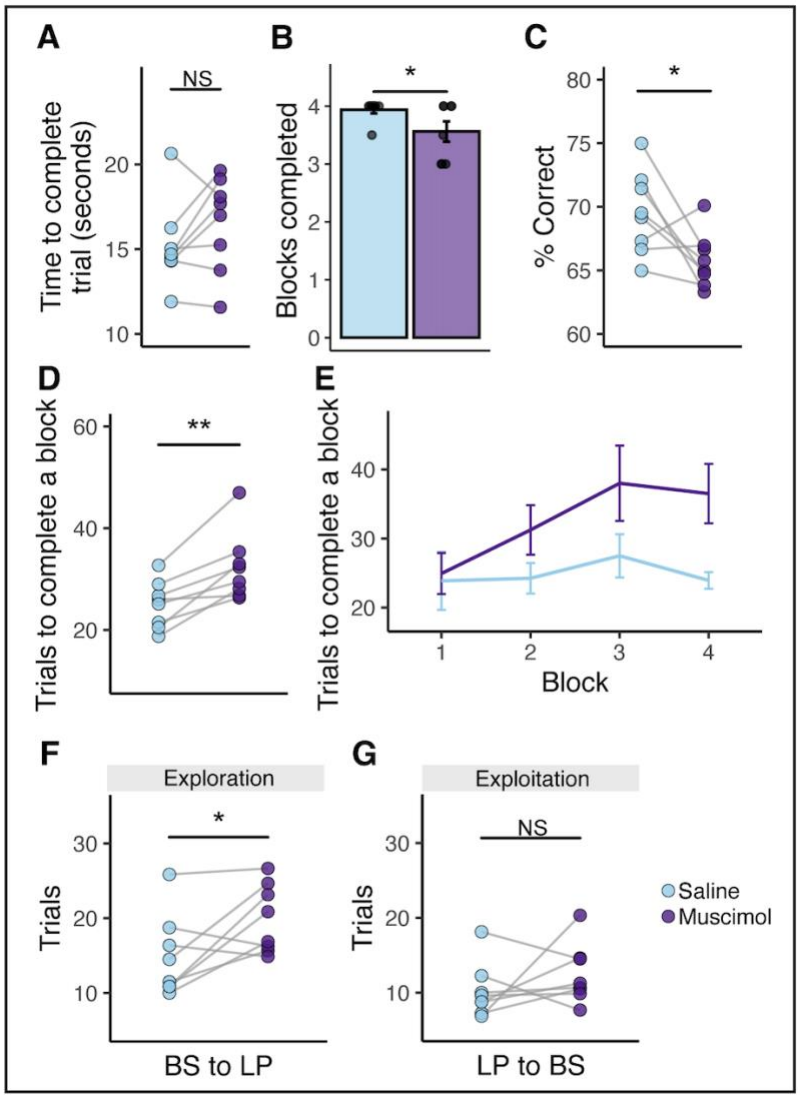


Figure 2.2

LHb inactivation impaired accuracy, especially during exploration. The time it took for rats to complete a trial was not different between conditions [A, LMM: $t(7) = -1.512$, $P = 0.174$]. Muscimol (represented in purple) resulted in a significantly lower average number of blocks completed by each rat compared to saline [B; LMM: $t(7) = 2.393$, $P = 0.048$; represented in blue]. Out of the completed blocks, the percentage of correct choices by condition is shown in C [LMM: $t(7) = 2.708$, $P = 0.030$], and the number of trials it took to complete a block is shown in D [LMM: $t(7) = -4.567$, $P = 0.003$]. When examined by block, trials to completion were not significantly affected by muscimol [E, LMM: block 1: $t(46) = -0.232$, $P = 0.817$; block 2: $t(46) = -1.531$, $P = 0.265$; block 3: $t(46) = -2.296$, $P = 0.079$; block 4: $t(47.3) = -2.535$, $P = 0.058$]. A deeper investigation of the trials by phase shows significantly more trials during exploration [F, LMM: $t(7) = -2.514$, $P = 0.040$], but not exploitation [G, LMM: $t(7) = -1.175$, $P = 0.278$], following LHb inactivation.

LHb Inactivation Decreases The Ability To Respond To Incorrect Choice Outcomes

As reward contingencies change, rats must evaluate their previous choices and outcomes to decide whether to maintain their current strategy or explore new alternatives, a process known to rely on the LHb (Nuno-Perez et al., 2021; Baker et al., 2017). This decision-making process naturally leads to errors, as rats might initially persist with the old strategy (Perseverative errors) or explore other alternative strategies. This study utilized the learning point as a marker for the adoption of a new strategy following a switch, as well as a transition point between error subtypes. Specifically, this study directly assessed total errors prior to the learning point, errors that occurred perseveratively (prior to the learning point, and increased the previous strategy likelihood), and errors that occurred regressively (following the learning point, and increased the previous strategy likelihood; Figure 2.3A). We found that LHb inactivation significantly increased total errors prior to the learning point (Figure 2.3B, left; $t(7)=-2.836$, $p=0.025$), but had no effect on the number of Perseverative (Figure 2.3B, middle; $t(7)=-1.723$, $p=0.129$), or Regressive errors (Figure 2.3B, right; $t(7)=-1.098$, $p=0.309$), when compared to saline-infused controls. Although total errors increased, the fact that errors following LHb inactivation did not lead to a reversion to the previous strategy indicates that flexible behavior was maintained, since rats were testing out other alternative strategy options.

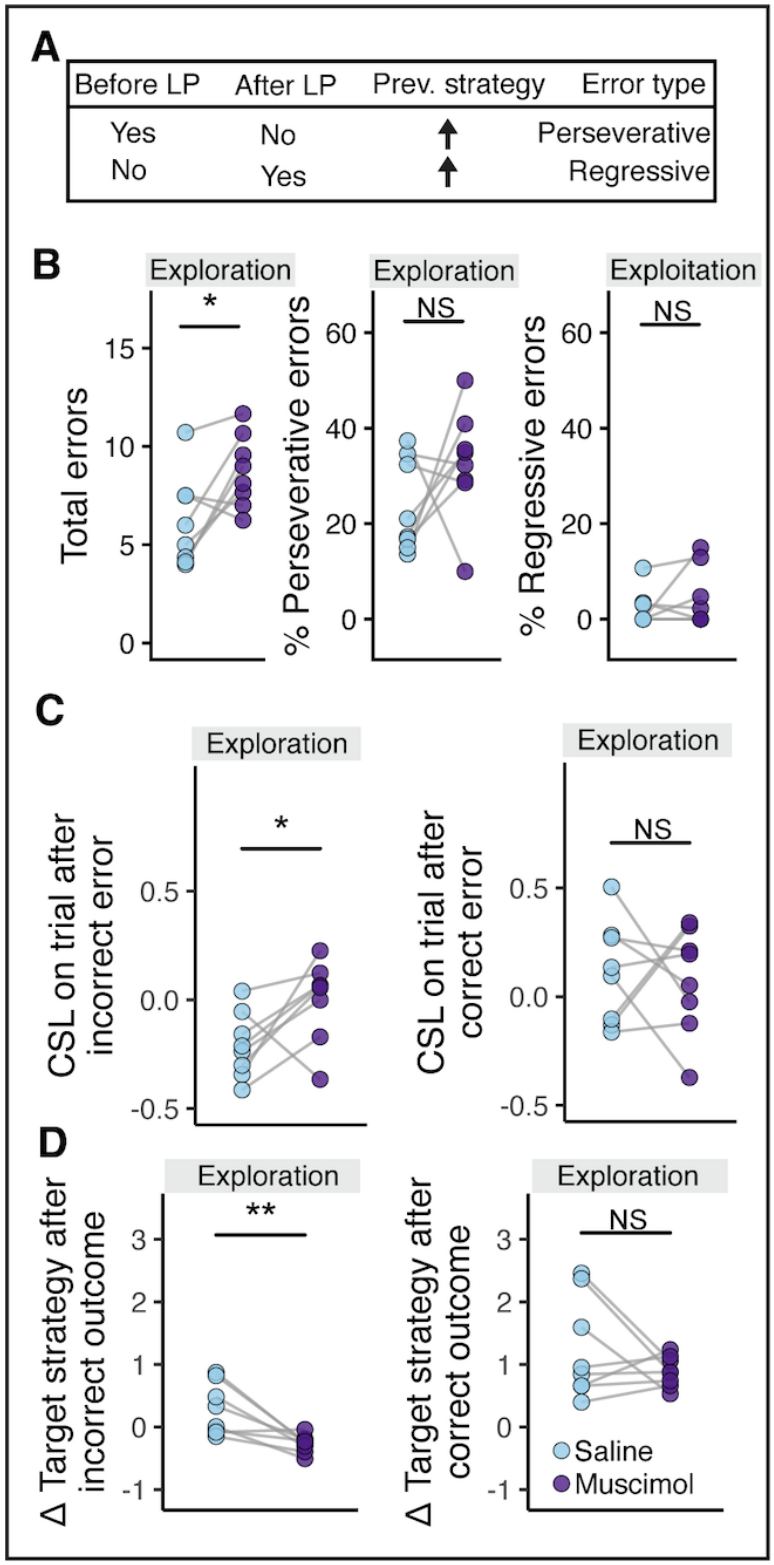


Figure 2.3

LHb inactivation impairs appropriate, adaptive responding following errors. A schematic representation of Perseverative and Regressive error calculation is shown in A. For instance, an error that occurred before the learning point and resulted in an increase in the previous strategy likelihood was classified as a Perseverative error. Alternatively, an error that occurred after the learning point and resulted in an increase in the previous strategy likelihood was classified as a Regressive error. LHb inactivation significantly increased total errors prior to the learning point [B, left, LMM: $t(7) = -2.836$, $P = 0.025$], but did not result in significant changes to the proportion of Perseverative [B, middle, LMM: $t(7) = -1.723$, $P = 0.129$], or Regressive [B, right, LMM: $t(7) = -1.098$, $P = 0.309$] errors. CSL on trials after incorrect [C, left, LMM: $t(7) = -2.487$, $P = 0.042$], but not

correct [C, right, LMM: $t(7) = 0.299$, $P = 0.774$], outcomes were significantly elevated during exploration. Median absolute deviations of target strategy changes following trial outcomes during exploration showed a significant decrease following incorrect [D, left, LMM: $t(7) = 3.660$, $P = 0.008$], but not correct [D, right, LMM: $t(7) = 1.210$, $P = 0.266$], outcomes.

The results so far demonstrate that LHb inactivation significantly increased errors prior to strategy learning (Figure 2.3B, left), but that errors during this phase of the block were largely not consistent with perseveration on the previous strategy (Figure 2.3B, middle). As such, we hypothesized that rats were still behaving flexibly, but not adaptively, when the LHb was inactivated. To test whether flexibility remained intact after LHb inactivation, the change in strategy likelihood (CSL; see Methods) was measured after errors and correct choices. To quantify the propensity to change strategies after errors, the CSL was compared on trials following correct and incorrect choices that occurred specifically between the block switch and learning point (exploration). CSL was significantly elevated following incorrect outcomes in the LHb inactivation condition, compared to the saline condition (Figure 2.3C, left; $t(7)=-2.487$, $p=0.042$), though this was not observed following correct outcomes (Figure 2.3C, right; $t(7)=0.299$, $p=0.774$). This result is consistent with error-driven flexibility not only remaining intact, but increasing during LHb inactivation.

Given that overall performance declined during LHb inactivation (see Figures 2.2, 2.3), and that flexible responding may not always be optimal, we tested how current target strategy likelihoods changed after errors and correct choices. We predicted that errors would lead to behavior consistent with the target strategy when the LHb was intact, indicating LHb involvement in adaptive strategy use. To test this prediction, changes in target strategy likelihoods were compared between trials, each with an incorrect or correct outcome, and the subsequent trial. The change in target strategy is a metric distinct from CSL, as these metrics differ in the types of strategy likelihoods used in the calculation (see Methods). Thus, a change in target strategy revealed whether or not the preceding trial's outcome resulted in a change toward the appropriate strategy (positive value; adaptive), or away from it (negative value; maladaptive). LHb inactivation resulted in a significantly reduced likelihood of using the target strategy following incorrect choices (Figure 2.3D, left; $t(7)=3.660$, $p=0.008$). Consistent with prior work demonstrating LHb sensitivity to negative outcomes (Matsumoto & Hikosaka 2007),

LHb inactivation did not change how rats adjusted strategies after correct choices (Figure 2.3D, right; t(7)=1.210, p=0.266). The finding that incorrect, but not correct, choices increase target strategy likelihoods when the LHb is intact is consistent with the hypothesis that the LHb contributes to adaptive responding by signaling the negative outcomes of choice.

Inactivation of The LHb Disrupts Task-Relevant Deliberative Behavior

Another proposed marker of behavioral flexibility and adaptability is vicarious trial and error (VTE; Miles et al., 2024; Redish, 2016). When examining the overall proportion of trials with VTEs during exploration and exploitation, VTEs only differed between conditions during exploration, not exploitation (Figure 2.4B; left: t(7)=3.081, p=0.018; Figure 2.4B right: t(7)=0.509, p=0.626). When examining VTE occurrences trial-by-trial, there were notable differences relative to important task events (Figure 2.4C). VTE rates were found to significantly decrease 9 (P=0.988, d=-2.127) and 10 (P=0.993, d=-2.287) trials before the block switch following LHb inactivation (Figure 4C, bottom left). Similarly, LHb inactivation abolished the usual peak in VTE activity 1 (P=0.997, d=-2.513) and 2 (P=0.992, d=-2.668) trials prior to the learning point (Figure 4C, bottom right). These results support the notion that LHb inactivation disrupts appropriately-timed VTE behaviors near task-relevant events, especially during the exploration phase.

Recent findings suggest that VTE can be adaptive when it occurs during periods of high flexibility (deliberative), where it tends to lead to correct choices; VTE may also reflect uncertainty when it occurs during periods of inflexible behavior (Miles et al., 2024). Given that the two trials prior to the learning point are automatically defined as deliberative (see Methods), and that these two trials were significantly reduced following muscimol (Figure 2.4C, right), we tested whether deliberative VTEs in particular contributed to the overall reduction in VTE rates during the exploration phase seen in Figure 4B. Indeed, we find that deliberative VTE rates were lower following LHb inactivation (Supplemental figure 2.1D; t(21) = 2.606, p=0.033). This was

not the case for uncertain VTEs (Supplemental figure 2.1D; $t(21) = 1.353$, $p=0.190$). These results suggest that an intact LHb contributes to adaptive usage of deliberative VTEs.

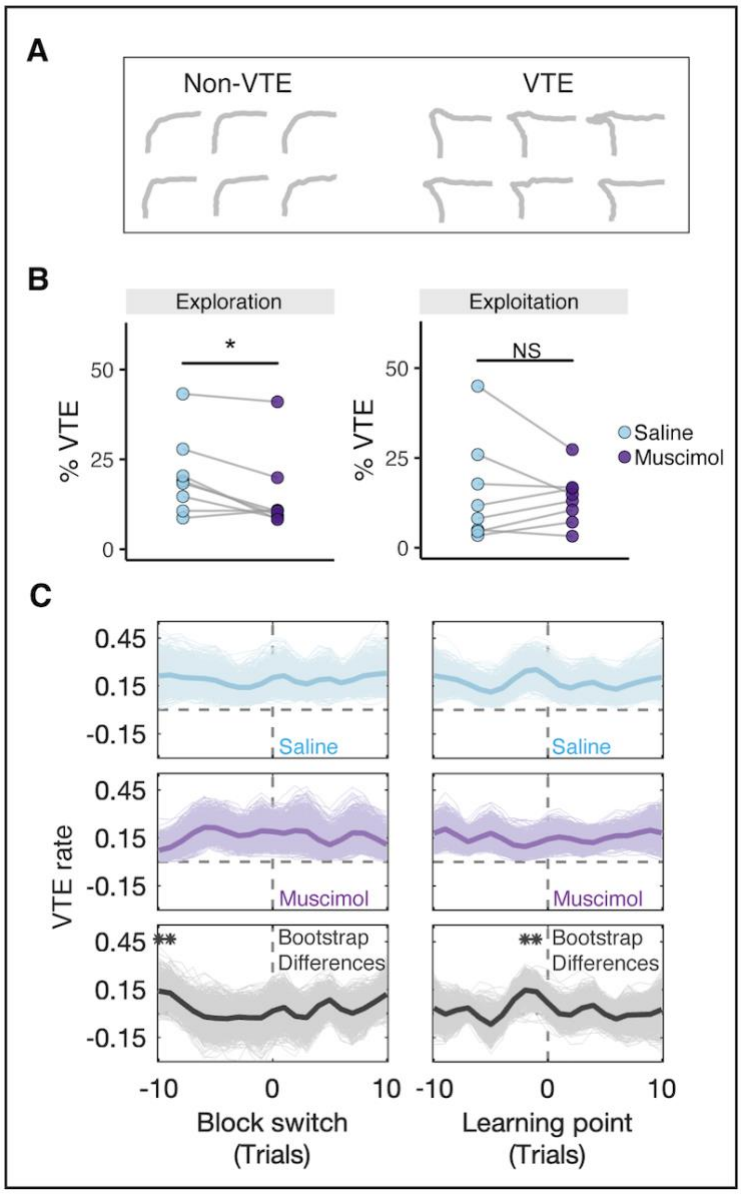


Figure 2.4

VTE rates are disrupted at task-relevant time points following LHb inactivation. Example representations of non-VTE and VTE trajectories aligned to start at the south start arm and terminate at the east reward arm (A). There was a significant decrease in VTE rates during exploration [B, left, LMM: $t(7) = 3.081$, $P = 0.018$], but not during exploitation [B, right, LMM: $t(7) = 0.509$, $P = 0.626$]. Bootstrapped VTE rates are aligned 10 trials before and after the block switch (C, left) and learning point (C, right). One thousand bootstrap iterations are plotted for saline in blue (C, top), and muscimol in purple (C, middle), with bold lines representing the averages. One thousand subject-wise paired bootstrap subtractions are plotted alongside the average in black (C, bottom). LHb inactivation suppressed VTE rates 9 (C, bottom left, $P = 0.988$, $d = -2.127$) and 10 (C, bottom left, $P = 0.993$, $d = -2.287$) trials prior to the block switch, as well as 1 (C, bottom right, $P = 0.997$, $d = -2.513$) and 2 (C, bottom right, $P = 0.992$, $d = -2.668$) trials prior to the learning point. Horizontal dashed lines represent VTE rates of 0, while vertical dashed lines represent the key task point (block switch or learning point).

Discussion

The findings described here complement and extend our growing understanding of the LHb's role in flexible decisions and adaptive behavior. Specifically, utilizing a complex spatial set-shifting task, this study found that inactivating the LHb with muscimol resulted in less ability to flexibly switch between learned strategies (Figure 2.2B), impaired overall performance (Figure 2.2C), and increased number of trials to complete a block (Figure 2D). These effects may have been driven by poorer performance during the later blocks (Figure 2.2E), suggesting that successive strategy switches might be increasingly challenging with an inactivated LHb, though the comparison of individual blocks was insufficiently powered to reach statistical significance.

Dissecting each block into two phases (block switch to the learning point (exploration) and learning point to the block switch (exploitation)), revealed additional details of the LHb's contribution to flexible decision making. Rats required more trials to reach the learning point following LHb inactivation (Figure 2.2F). However, once the target strategy was adopted (Figure 2.2G), there was no difference between muscimol or saline treated rats. This suggests that the LHb is selectively important for the process of adapting to a different, appropriate strategy following a rule shift.

Interestingly, LHb inactivation increased exploratory behaviors (as evidenced by enhanced flexibility, or CSL) while at the same time reduced the use of the target strategy following incorrect, but not after correct, choices (Figure 2.3D). This pattern of effects supports the LHb's role in appropriately using negative choice outcome information to adaptively inform future behaviors, in line with earlier negative reward prediction error literature (Matsumoto & Hikosaka, 2007; Bromberg-Martin & Hikosaka, 2011; Tian & Uchida, 2015; Li et al., 2019). Finally, LHb inactivation impaired vicarious trial and error, or VTE, behaviors near task-relevant key points (Figure 2.4C), with this effect most clearly observed for deliberative VTEs (Supplemental Figure 1E), particularly during the exploration phase (Figure 2.4B). The VTE findings further support the notion that the LHb is important for adaptive decision-making.

The LHb supports the adoption of appropriate strategies

The LHb guides disengagement of prior behaviors and adaptation of appropriate strategies

The ability to discriminate between optimal and suboptimal behaviors towards a given goal is crucial for decision-making, and is itself a type of behavioral flexibility. Once a behavior is no longer deemed optimal, animals must promptly disengage and begin exploring alternative strategies. Recent research has implicated the LHb in driving behavioral disengagement (Proulx et al., 2018; Stamatakis & Stuber, 2012; Mondoloni et al., 2022). For instance, inactivation of the LHb resulted in prolonged reward-seeking behavior and disengagement from this behavior was characterized by increased tonic activity in the LHb (Post et al., 2022). By reversibly inactivating the LHb on a set-shifting task, the present study showed that rather than impairing disengagement from prior behaviors, the ability to adaptively select the new, optimal behavior was impaired. This was evidenced by the increase in flexibility (Figure 2.3C, left), reflecting attempted changes in behavior, but a decrease in the use of the target strategy (Figure 2.3D, left) following errors during exploration. Importantly, these effects were not seen once the target strategy was adopted (Figure 2.2G), which is in agreement with earlier literature showing that the LHb selectively contributes to early learning (Baker et al., 2019; Mathis & Lecourtier, 2017; Mathis et al., 2015). Our findings suggest that the LHb contributes specifically to the adoption of optimal strategies when contingencies change.

Given the LHb's known role in aversive coding (Matsumoto & Hikosaka, 2007; Hikosaka et al., 2008; Bromberg-Martin et al., 2010), the inability to effectively select the appropriate strategy following inactivation could be attributed to either the improper evaluation of, or decreased sensitivity to,the previous choice's negative outcomes. Since we see increased changes in strategy usage during LHb inactivation, our results argue against the latter interpretation. Furthermore, prior to the learning point, the decrease in target strategy use after incorrect, and not correct, choice outcomes following LHb inactivation suggests that rats were inappropriately

responding to negative choice outcomes (Figure 2.3D). This might suggest a reduced ability to adaptively use negative choice outcomes to inform future behavior, resulting in increased trials during the selection of more optimal strategies. In summary, inactivation of the LHb, and its purported "brake signal" (Post et al., 2022; Desrochers et al., 2022), results in an increase in flexible, albeit maladaptive, behavior that impairs performance.

The LHb supports task-relevant deliberative behavior

VTE is a behavior characterized by pausing and looking at potential options at the choice point, a behavior attributed to either deliberation or uncertainty (Miles et al., 2024; Redish 2016; Porter et al., 2025). Manipulations that impair task performance often also affect VTEs (Kidder et al., 2024; Kidder et al., 2021; Hu & Amsel, 1995; Blumenthal et al., 2011). The present study showed that LHb inactivation resulted in an overall reduction in VTE rates during the exploration phase (Figure 2.4B), a decrease in VTEs 9 and 10 trials prior to the block switch (Figure 2.4C, left), as well as the attenuation of the characteristic peak in VTEs 1 and 2 trials before the learning point typically seen in control rats (Figure 2.4C, right). These results mirror those seen in another study disrupting the medial prefrontal cortex (mPFC) on a spatial reversal learning task in that VTEs were decreased surrounding critical moments in the task, though overall rates in that study were not affected (Kidder et al., 2024). This similarity is particularly notable given the overlapping functions of the LHb and mPFC and their interdependence in supporting appropriate decision-making (Mathis et al., 2017; Capuzzo & Floresco, 2020).

Given that VTE behaviors tend to increase with challenge (Amemiya et al., 2014; Schmidt et al., 2019), the finding that LHb inactivation suppresses task-relevant VTEs suggests LHb activity may normally promote deliberative and uncertainty-related behaviors, potentially by generating aversive signals that enhance deliberation and uncertainty during decision-making. Importantly, muscimol significantly reduced VTE rates, with effects most clearly observed during deliberative trials (Supplementary Figure 2.1D). In support, deliberative VTE rates were impaired 1 and 2 trials before the learning point (Figure 2.4C, right). This suggests

that deliberative VTEs, specifically, facilitate behavioral adaptation to new strategies and are supported by a functional LHb. Although we can confidently state that deliberative behaviors are impaired right before the learning point when the LHb is inactivated, the VTE impairments surrounding the block switch are harder to explain given how far they are from the block switch itself. This is in part due to the fact that learning points can occur as early as 7 trials after the block switch, or as early as 7 trials before the block switch. Nevertheless, the fact that VTE timing is disrupted relative to typical task features (Figure 2.4C) may reflect an underlying failure to properly evaluate and integrate sensory, motivational, and contextual cues.

Outcome evaluation in the LHb

Evaluation of response outcomes is an important integrative process that supports behavioral flexibility by incorporating a variety of internal and external cues. An important aspect of evaluating a response outcome is the generation of reward prediction errors (RPE), which are signals representing errors in predicting the value of primary rewards. Such signals contribute to the updating of action-outcome associations and guide future decision-making. RPE signals are often attributed to dopaminergic neurons (Hikosaka et al., 2008). However, a seminal study by Matsumoto & Hikosaka (2007) found the LHb to play a critical role in the RPE signaling cascade. Given the LHb's anatomical and functional connection with both limbic and midbrain structures, it functions as a critical integrative hub to inform future action (Christoph et al., 1986; Herkenham et al., 1977; Herkenham et al., 1979; Hones & Mizumori, 2022). Although identifying and examining traditional RPEs in the LHb was not the primary goal of the current study, LHb inactivation paired with the spatial set-shifting task offers additional insight into LHb's behavioral contribution following negative events. The present study reveals that the function of the LHb encompasses more generally the integration of outcome evaluation during adaptive strategy switching. That is, higher CSL values following incorrect trials during LHb inactivation sessions may reflect the increased propensity to change choice behavior following a negative outcome. Interestingly, the increase in CSL following LHb inactivation (Figure 2.3C,

left) did not result in adaptive target strategy use (Figure 2.3D, left). This is in line with the idea that flexible responding may not always result in the use of the optimal strategy (Lea et al., 2020). Thus, during exploration, an intact LHb is required to utilize negative choice outcome information to guide adaptive decision-making. By doing so, LHb effectively reduces response flexibility per se, which in turn reduces the likelihood of making suboptimal decisions.

The results of the present study deviate from previous literature showing increased Perseverative errors following LHb inactivation (Baker, Raynor, Francis, & Mizumori, 2017). Likewise, our study failed to reproduce the effects of LHb inactivation on Regressive errors that were reported in a previous study (Figure 2.3B; Baker et al., 2015). This is likely a function of the use of a different error calculation and the reliance on indirect error definitions. For instance, Baker and colleagues (2015) used 2 correct choices on a given block as the switch point between Perseverative (before 2 correct choices) and Regressive (after two correct choices). Although two errors may provide a rough estimate of strategy adaptation, it only serves as a proxy. The error calculations in the present study rely on the strategies that the rats are using on a trial by trial basis, along with a clear point in the task when the target strategy has been adopted, marked by its likelihood relative to other strategies. This offers a more direct estimate of errors that are a result of perseverating on the previous block's strategy prior to the learning point, as well as errors that are a result of regressing to the previous block's strategy after the learning point. With these clear definitions that satisfy features of the behavior that have been difficult to address, the current study found no significant effects of LHb inactivation on Regressive or Perseverative errors.

Although few studies have reported sexual dimorphism relative to the LHb on similar tasks (Baker et al., 2019; Sevigny et al., 2021; Wright et al., 2025), there are notable anatomical and structural differences between the sexes in rodents which may contribute to dissociable function and effects of manipulation (Liu et al., 2022). These important potential influences of sex on LHb function were not assessable in our limited sample size. Further studies will be

needed to address potential sex differences, as well as functional and anatomical projections associated with the LHb to identify the circuit mechanisms that support appropriate behavioral adaptation.

The LHb may enable a hippocampal neural state to support flexible decision-making

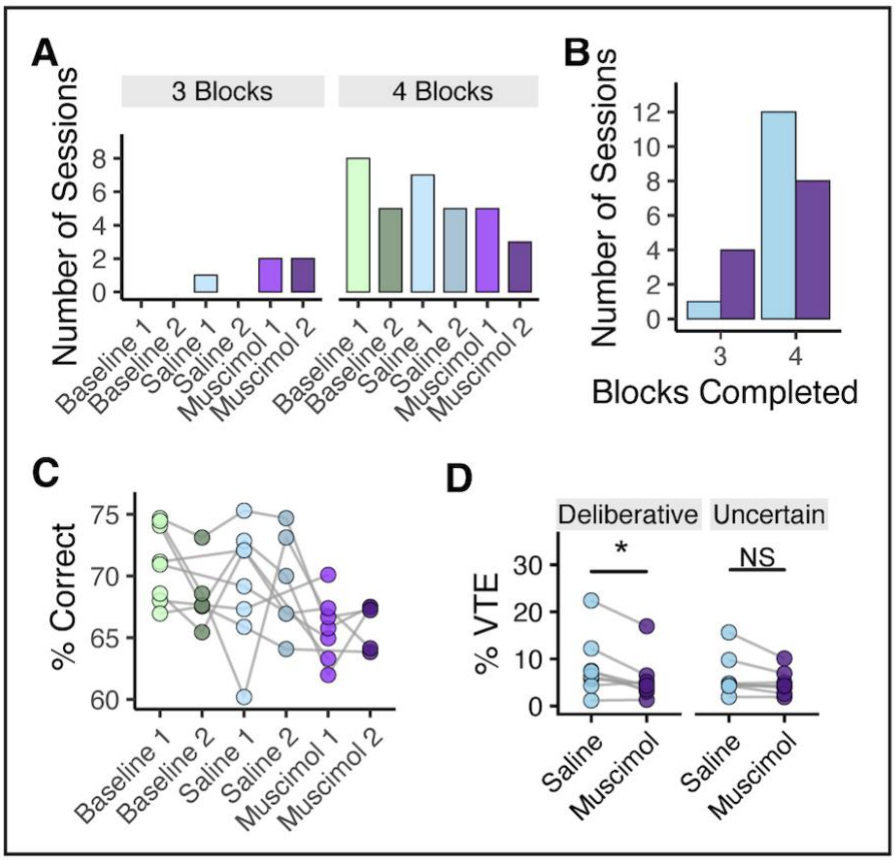
The results of this study reveal new information about how the LHb contributes to adaptive responding, which broadens our understanding of its role within a larger neural system that supports flexible behavior. As proposed by Hones and Mizumori (2022), the LHb not only projects to the classical monoaminergic structures (e.g., ventral tegmental area, dorsal raphe) to select and initiate actions, but it also sends critical information about ongoing behaviors to loci such as the locus coeruleus, supramammillary nucleus, median raphe, and the nucleus incertus. These structures are known to modulate hippocampal (HPC) theta oscillations, a key physiological mechanism widely associated with attention, context processing, and response flexibility (see review by Hones & Mizumori, 2022).

In support of a hypothesized HPC-LHb interaction, earlier work found that LHb spiking activity couples to HPC theta, and lesioning the LHb resulted in HPC theta impairments (Aizawa 2012). Another study found that ipsilateral and contralateral inactivations of the LHb and HPC led to impaired performance on a spatial task, linking the interaction between the two structures to response flexibility (Baker et al., 2019). In addition to the LHb, a structure that is commonly attributed to driving flexible strategy switching is the prefrontal cortex (PFC; Avigan et al., 2020). Given that there are direct anatomical projections between the medial PFC and the LHb (Kim & Lee, 2012), the LHb may integrate contextual and memory information from the mPFC, enabling appropriately timed behavioral responses. Indeed, studies chemogenetically inactivating the LHb and mPFC simultaneously resulted in decreased ability to perform context-based memory tasks (Mathis et al., 2017; Kim & Lee, 2012).

In the present study, LHb inactivation reduced the ability to shift towards the target strategy following negative outcomes (Figure 2.3D), which may reflect impaired integration of contextual and motivational cues that guide behaviors. By relaying signals regarding negative outcomes, the LHb may set a neuromodulatory tone that alters the functional and neural state of the hippocampal circuit (Bueno et al., 2019; Hones & Mizumori, 2022). This interplay likely facilitates the transition from one behavioral state to another, ensuring that learning and memory processes dynamically adjust to evolving contingencies. Our results add to a growing body of evidence underscoring how coordinated activity between the LHb and a broader circuit of midbrain and limbic nuclei supports the fluid modification of behavioral strategies. These findings have a broader relevance to human psychiatric conditions, such as depression, anxiety, and addiction, which are often characterized by maladaptive and inflexible decisions (Stange et al., 2017; Verdejo-Garcia et al., 2015).

Conclusion

In summary, inactivating the LHb reduced strategy switching, impaired overall task performance, and increased the number of trials required to complete a block. These effects were driven by a dampened adaptation to new strategies during exploration, as muscimol-treated rats took longer to reach the learning point, while performance during exploitation remained intact. Inactivation also diminished the use of negative choice outcomes to guide future strategy use and disrupted VTEs at task-relevant time points. Together, these findings extend our understanding of LHb functioning by suggesting that negative or aversive signaling by the LHb contributes to adaptive strategy switching and flexible decision-making in appetitive situations.



Supplementary Figure 2.1

There were no notable differences between the first and second infusions in either muscimol or saline conditions (A-C). When comparing all 6 sessions of the experimental paradigm (2 baseline, 2 saline, 2 muscimol), rats completed fewer blocks under both muscimol sessions (3.6–3.7) compared to baseline and saline (~4.0), with the reduction most evident in Muscimol 2 (A, LMM: t(27) = −2.307, p = 0.029). As a result, both infusions of saline and muscimol were combined and represented in B. Similarly, choice accuracy within completed blocks was most impaired with muscimol (C, Muscimol 1 LMM: t(27) = -3.398, p = 0.002, Muscimol 2 LMM: t(27) = -2.771, p = 0.009). *LHb inactivation did not affect the subtypes of VTEs, but did increase flexible responding exclusively following incorrect choices during exploration*. Deliberative VTEs were more frequent than uncertain VTEs across conditions (D, LMM: t(23) = 3.423, p = 0.002) with no effect of muscimol (D, t(23) = 0.781, p = 0.443).

Materials & Methods

Subjects

This study was performed in accordance with the guidelines established by the National Institutes of Health and approved by the University of Washington's Institute for Animal Care and Use Committee. Eight 3-month old Long-Evans rats (female = 6, male = 2; 250–350g; Charles River Laboratories) were used to carry out this experiment. Rats were single-housed in a temperature-controlled environment with a 12-hour light/dark cycle. Training and experiments were performed during the light phase. Prior to training, rats were handled for a minimum of 10 minutes for five consecutive days and food restricted to 85% of their free-feeding body weight for the training paradigm and experiment.

Apparatus & Training Procedures

The apparatus and training procedures were similar to Miles et al., 2024. In brief, an elevated plus maze composed of plexiglass (arms = 58 cm × 5.5 cm, height = 80 cm) was utilized for the fully automated spatial set-shifting task. The maze was surrounded by black curtains with spatial cues, and located in a sound-attenuated room to minimize distractions. Experimenters were in an adjacent room monitoring rat behavior via a custom LabVIEW program (2016; National Instruments, Austin, TX). The LabVIEW program used an Arduino to raise and lower the portion of maze arms proximal to the center platform by tracking the rats' position using a SONY USB camera (~35 fps). The maze consisted of a center platform, two arms extending East and West, and two arms extending North and South. 3-D printed reward ports were located at the ends of the East and West arms, while the North and South arms (pseudo-randomly selected to prevent the use of an egocentric strategy) served as the start locations for each trial, and the locations rats returned to after executing a choice and receiving reward feedback. Rats remained on the return arm for a variable two to five second delay before

being able to initiate a new trial. Both potential reward locations were available to choose on every trial.

The set-shifting task consisted of three strategies: place-West, place-East, and Alternation. For each of the place strategies, rats were required to consistently select the respective reward arm on each trial (i.e. West or East). The Alternation strategy required the rats to alternate between the West and East reward arm every trial in order to obtain reward. Initial training began with maze habituation, where rats were exposed to the maze for 10 minutes for two to three consecutive days. Rats were then pre-trained on a simplified version of the task consisting of three forced choice blocks, one for each strategy (place-East, place-West, Alternation), in which a single choice arm was raised forcing the rat to take that path. At the end of each trial, rats received a sucrose pellet reward (45mg; TestDiet, Richmond, IN, USA). The length of these blocks increased session-by-session as rats improved, starting at five trials per block (15 trials total), and ending at 15 trials per block to complete 45 trials in 60 minutes. After completion of pre-training, rats were introduced to a free-choice version of the task, where both arms were available to choose on every trial. Here, a session was composed of a randomly generated sequence of block types, consisting of two Alternation blocks and one each of place-East and place-West blocks. This sequence came with the condition that the two Alternation blocks could not be ordered back-to-back. Rats began on a randomly presented block type and had to deduce the correct strategy through trial-and-error. In order to advance from one block to the next (marked by a change in reward contingencies), rats were required to complete 12 correct responses out of a rolling 15 trial window. Rats qualified for surgery upon completing three strategy switches, or four blocks, within 60 minutes for two consecutive sessions.

Surgery

Once rats reached criterion on the spatial set-shifting task, food-restriction was discontinued, allowing the rats to regain their free-feed body-weight for one to four days in

preparation for surgery. Anesthesia was induced with 4% isoflurane (mixed with oxygen at a flow rate of 1 L/min) in an induction chamber. The rats were then mounted in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). Subsequently, the isoflurane concentration was reduced to 1%–3% to maintain the desired anesthetic depth. Eight craniotomies were performed, of which two were used to bilaterally target the LHb (AP: -3.6 mm, ML: +/- 0.8 mm, DV: -4.8 mm; top of skull, relative to Bregma), and the rest were used for anchor screws. Bilateral guide cannulae (26 gauge; Protech International) were lowered at 100 µm per second to a depth that was 1.2 µm dorsal to the LHb (the injection needle extended 1.2 µm past the guide cannula). Dental cement adhesive was applied to the skull surrounding the implant and screws to secure the guide cannula. Internal dummy cannulas were inserted into the guide cannula to prevent clogging and secured with a dust cap (Protech International). Following a seven-day post-surgery recovery period, the rats were reduced back down to 85% of their ad libitum free-feeding weight and re-trained on the task.

Histology

Following the completion of the experimental paradigm, rats were deeply anesthetized with 4% isoflurane and were overdosed using sodium pentobarbital (1-2 mL/kg, I.P.). Immediately after, 250µL of DiI Cell-Labeling Solution (ThermoFischer) was infused into each hemisphere of the LHb, to confirm targeting, at a rate of 0.15µL/min. To prevent backflow and ensure proper absorption of the solution, cannula were removed after a 90-second post-infusion waiting period. Rats were then transcardially perfused with 150mL of phosphate buffered saline, followed by 150mL of formaldehyde solution (4% formaldehyde). Bilateral DiI expression was observed in the LHb (Figure 2.1B, top) using a fluorescent microscope.

Experimental Design & Statistical Analyses

Experimental paradigm

To temporarily inactivate the LHb, the GABAa receptor agonist muscimol was infused into the LHb. The experimental paradigm extended across six days, with four counterbalanced muscimol and saline infusion days in a blinded ABBA format (days 2 through 5), and two baseline days on days 1 and 6, with the exception of three rats (all females) that underwent an unblinded single baseline, muscimol, and saline infusion day, as well as one rat (female) that underwent a single muscimol infusion day. While being held by the experimenter, awake rats received 250nl of muscimol (26-35ng/250nl) or saline (0.9%) infusions at a rate of 0.15µl/min in each hemisphere of the LHb. Each infusion was followed by a 90 second waiting period with the infusion needle in place. After this waiting period, the needle was removed, and the rats were placed back in their home cage for an additional 2.5 minutes before being introduced to the task, resulting in a total wait time of 5 minutes between infusion and task engagement. During baseline sessions (Days 1 and 6), the needle remained in place without infusion for 1 minute before removal, followed by a 4-minute waiting period prior to task placement.

Quantifying strategy likelihoods and other task metrics

The likelihood that a rat was using one of the three available strategies (place-West, place-East, or Alternation) was quantified as described in Maggi et al., 2024 and Miles et al., 2024. Using the success and failure of each strategy type on each trial (e.g. if a rat went West, it was a success for the go West strategy, and a failure for the go East strategy), recency-weighted bayesian inference resulted in estimates of the likelihood that a rat used each strategy based on their recent choices (Figure 2.1C). The 'learning point' within each block was identified as the first trial in which the target strategy became the most likely one used by the rat for the remainder of the block, while the 'block switch' represented the trial at which the strategy rule switched and reward contingencies changed. In this study, trials after the block switch but prior

to the learning point represented the behavioral adaptation to the new target strategy following an uncued switch (exploration), while trials after the learning point but prior to the block switch reflected consistent use of the newly acquired target strategy (exploitation).

While all three strategy likelihoods were calculated for salient task measures, the propensity to switch strategies, termed a 'change in strategy likelihood' (CSL), was quantified by first calculating the trial-by-trial magnitude change in the Alternation strategy likelihood and then normalizing by session using the median absolute deviation. A higher CSL indicated that a rat was more likely to change its strategy between trials, whether that was towards one strategy or away from another. This calculation deviates from the 'flexibility' calculation described in Miles et al., 2024 in that only a single type of strategy likelihood was used for the calculation as opposed to all three. Since the Alternation likelihood increases during Alternation blocks and decreases during Place blocks, the calculation was simplified to rely only on one strategy, representing the adaptability of the rats' choice behavior from trial-to-trial.

Position tracking, VTE classification, and bootstrapping

During moments of uncertainty, such as when reward contingencies change, rats exhibit behavioral uncertainty in the form of vicarious trial and errors (VTE; Schmidt et al., 2013; Redish, 2016). Studies have shown that VTEs support flexible decision making (Kidder et al., 2024), and decreased VTE rates correlate with poor performance (Kidder et al., 2021). Here, VTEs were identified as described in Miles et al., 2024 and Kidder et al., 2024. Briefly, pose-estimates for the head were generated using DeepLabCut (version 2.2; Mathis et al., 2018). The model was trained on additional data using NVIDIA GEFORCE GTX 1080 GPU with 500,000 iterations. Position data were then projected into principal component space, and trajectories were categorized into VTE and non-VTE clusters using a custom MATLAB script.

VTE behavior was aligned in MATLAB (version 2023b) across 10 trials immediately before and after 2 key task-relevant events—the block switch and learning point. To estimate behavioral patterns surrounding these events, a hierarchical bootstrapping procedure was

applied at the subject (N=8) and block level (N=4 for block switches; N=6 for learning point) for 1,000 iterations, providing a robust estimate of behavioral patterns surrounding the block switch and learning point. Each bootstrap iteration was pairwise subtracted, within each rat, for the saline and muscimol conditions, resulting in subject-level differences between conditions. P values were calculated as the proportion of bootstrap difference iterations for a single trial that fell above or below zero, while d values were calculated as the standard deviation from the mean. A P value above 0.975 or below 0.025 combined with a d value of magnitude 1.5 or greater was considered to be significant, meaning that 97.5% of the iterations for a single trial fell above or below zero, with zero indicating no difference between samples.

To test whether LHb inactivation led to differences in VTE usage, VTEs were classified as either deliberative or uncertain as outlined in Miles et al., 2024. Briefly, deliberative VTEs occurred during trials at the 60th percentile and above of CSL scores or two trials before or after the learning point, and could not occur within 3 trials of the block switch (unless they were within two trials of the learning point). Uncertain VTEs occurred during trials at the 40th percentile and below of CSL scores that did not border the learning point.

Error quantification

Errors were classified as Perseverative when two criteria were met: the error occurred prior to the learning point on a given block, and the previous block's strategy likelihood increased between the error trial and the subsequent trial. This reflects continued use of the preceding strategy prior to adopting the target strategy of the current block. Regressive errors occurred after the learning point on a given block, and increased the previous block's strategy likelihood between the error trial and the subsequent trial. These definitions are consistent with choice behavior representing perseveration of past strategy use prior to learning the new target strategy, and regressing to the prior strategy once the new target strategy has been adopted.

Statistical Analysis

For non-bootstrapped statistical testing, a linear mixed-effects model analysis was used in R (lme4 package; version 2023.09.0), which included the condition (averaged across sessions) as the fixed effect and the subject (rat) as the random intercept to account for repeated measures within subjects. Post-hoc pairwise comparisons between conditions were conducted using estimated marginal means with Tukey's method to control for multiple comparisons. Statistical significance was defined at $p \leq 0.05$.

Chapter 3

The role of the medial prefrontal cortex in adaptive strategy switching & object discrimination during a maladaptive state

Introduction

Flexible and adaptive behavior depends on an animal's ability to modify its behavior as the world around them changes. A rat foraging in a forest and a person navigating a new job face the same fundamental challenge: behaviors that worked yesterday may no longer work today. The ability to know *when* to shift our behavior, as well as the actual shifting of the behavior, is defined as behavioral flexibility. Importantly, modifying our behavior is not always optimal; often, it is beneficial to maintain our current behavioral strategy, or to be rigid, in the case that the strategy continues to result in positive outcomes. Likewise, in an attempt to be flexible and modify our behavior, we might elect to utilize a strategy that is suboptimal and no better than the strategy we were using previously, resulting in maladaptive behavior. However, in the instance that we are flexible and choose a behavioral strategy that is more ideal than the one we were previously engaged in, as well as manage to maintain that strategy, such a flexible behavioral shift is deemed adaptive. Flexibility and adaptability draw on a constellation of cognitive processes that span working memory, reward valuation, and the incorporation of motivational and contextual information to inform future decisions (Ragozzino et al., 2007; Capuzzo & Floresco, 2020; Kidder et al., 2024). When any of these processes fail, animals can become locked into outdated strategies, unable to disengage from choices that are no longer beneficial, or animals can continuously shift from strategy to strategy, despite the adaptive need to be inflexible. Such maladaptive decisions are common pathological behaviors in many psychiatric conditions.

Many psychiatric conditions are defined, at least in part, by impairments in behavioral flexibility (Stange et al., 2017; Giovanniello et al., 2023). Those with depression often express feeling trapped in negative thought patterns, individuals with obsessive compulsive disorder repeat behaviors despite the absence of any reinforcing outcome, and people with substance use disorders continue to seek and use drugs even as the rewarding aspects of the drug diminish (Morris & Mansel, 2018). These maladaptive choice patterns are a manifestation of dysfunction within the neural circuits that typically support flexible and adaptive behavior. The medial prefrontal cortex serves as one of the main structures contributing to these functions (mPFC; Goldstein & Volkow, 2011; Xu et al., 2019). Understanding how the mPFC contributes to flexible behavior, as well as how its function is altered in maladaptive states, is necessary for our understanding of the neural underpinnings of psychiatric disorders.

The mPFC is known for its central role in flexible decision making in humans and non-human animals. Lesion (de Bruin et al., 1994; Sapiurka et al., 2016), as well as electrophysiological and optogenetic studies (Warden et al., 2012) have shown that the mPFC is required for animals to appropriately update their behavior when task rules or reward contingencies change. Within the rodent mPFC, the prelimbic subregion in particular has been linked to the updating and maintenance of task rules (Rich & Shapiro, 2009). At the single-neuron level, prelimbic neurons have been shown to track the value of rewards, but only when choice behavior is involved (Sackett et al., 2019), suggesting the representation of the costs and benefits associated with future choices.

Given the implication of the mPFC in flexible behavior and reward valuation, it is no surprise that this structure plays a significant role in opioid use disorder. Chronic opioid use results in impairments in cognitive flexibility and working memory, and these deficits persist even after extended periods of abstinence (Halbout et al., 2024). Studies following chronic opioid exposure have revealed structural and functional alterations within the mPFC, including reductions in dendritic spine density (Robinson et al., 2002), as well as increased activation

(Piao et al., 2017). These prefrontal alterations have been proposed to contribute to the high rates of relapse seen in opioid use disorder: the mPFC that cannot flexibly update its responses may fail to override drug-seeking behavior in the presence of negative outcomes (Kalivas & Volkow, 2005). The mPFC, then, is a critical node in the circuit for understanding the cognitive impairments that accompany opiate addiction and withdrawal.

Several theories have been proposed to explain how opioid withdrawal disrupts mPFC function and produces its characteristic cognitive impairments. One such theory in the literature discusses how chronic opioid exposure shifts the excitation-inhibition balance within the mPFC, producing lasting changes in the excitability or prelimbic neurons and distorting the signaling of task-relevant information. In support, using electrophysiological recordings, Mohammadzadeh and colleagues (2022) showed that chronic morphine administration reduced the activity and functional connectivity of neurons in the mPFC across all frequency bands, and that this suppression rebounded into hyperactivity following the onset of withdrawal. Complementing this finding, other studies have shown that morphine withdrawal increases the intrinsic excitability of thick-tufted (Drd2+) but not thin-tufted (Drd1+) pyramidal neurons in the prelimbic area of the mPFC (Kokane et al., 2023), suggesting a distinct cell-type specific vulnerability. Importantly, both types of these pyramidal neurons within the prelimbic area project to the nucleus accumbens (or ventral striatum), which serves as a key structure in encoding the rewarding effects of drugs (Kourrich et al., 2015). Consistent with the idea that opioid withdrawal produces an aberrant prefrontal state, Bassareo and colleagues (1995) showed that chronic morphine administration elevates basal extracellular dopamine in the prefrontal cortex, and that this elevation is further amplified following precipitated withdrawal. Together, these findings converge on the view that opioid withdrawal induces a hyperactive state within the mPFC, specifically dysregulating the activity of prelimbic neurons.

Another key player within the mPFC is the serotonergic system. The mPFC receives dense serotonergic input from the dorsal raphe nucleus and expresses high levels of serotonin

2A and 1A receptors (Santana et al., 2004), both of which have been linked to flexible decision making (Alvarez et al., 2021). Additionally, release of serotonin and exogenous serotonin receptor agonists in the mPFC suppress neuronal firing (Tian et al., 2016; Elliott et al., 2018; Morgan et al., 2023). These pharmacological manipulations result in changes in behavior. For instance, Morgan and colleagues (2023) optogenetically stimulated the dorsal raphe pathway to the mPFC and found improved strategy switching, while Floris and colleagues (2024) found that a single dose of psilocybin reduced heroin seeking in addicted rats.

Interestingly, chronic opioid exposure and withdrawal also disrupt the serotonergic system. Pomrenze and colleagues (2022) showed that during opioid withdrawal, dynorphin released from dorsal raphe neurons activated kappa opioid receptors on serotonergic terminals in the nucleus accumbens, thereby suppressing local serotonin release and producing pathological behavioral changes. Currently available treatments for opioid use disorder do little to address this serotonergic dysfunction directly. Opioid replacement therapies such as buprenorphine and methadone substitute one opioid agonist for another and, while effective at managing withdrawal symptoms, come at a cognitive cost. Those that are on long-term opioid maintenance therapy show impairments in executive function, cognitive flexibility, as well as increased oxidative stress (Soyka et al., 2008; Arezoomandan et al., 2022). Interventions capable of restoring typical serotonergic functioning or promoting plasticity within the mPFC may be able to reverse at least some of the cognitive deficits following chronic opioid exposure. Consistent with this hypothesis, recent work has shown that a single dose of psilocybin, a potent serotonin 2A receptor agonist, results in rapid and long lasting increases in dendritic spine density, dendritic size, and synaptogenesis in the mPFC (Shao et al., 2021). Thus, the serotonergic system may be an important therapeutic target to reduce the unwanted consequences of opioid withdrawal.

Flexible adaptation requires access to contextual information to inform future decisions. The present location of an animal, the memory of the events that have taken place in that

location, as well as the choices that have previously been rewarded under similar contexts are all necessary to appropriate decision making. Contextual information has long been associated with the hippocampus (HPC), which represents the spatial and temporal features of an environment (Morris et al., 1982). For this contextual information to influence ongoing flexible decision-making, it necessitates the HPC and mPFC to work in concert to integrate outcome expectancies and rules as well as contextual representations (Dolleman-van der Weel et al., 2019). The ventral hippocampus sends dense projections to the prelimbic cortex (Hoover & Vertes, 2007), and simultaneously inactivating the mPFC and HPC impairs spatial memory processes (Churchwell & Kesner, 2011). In another study, simultaneous electrophysiological recordings from the HPC and mPFC in rats performing a context-guided object memory task showed that information flowed from the HPC to the mPFC upon entry into the context and reversed direction during object sampling (Place et al., 2016). These studies suggest that the HPC and mPFC communicate important context-related information during complex tasks.

The comparatively simple novel object recognition test (NORT) offers another way to probe the mPFC and its contribution to contextual processing. The task probes the ability of animals to remember the objects that they have previously encountered in a given environment and to direct their innate novelty-seeking exploratory behavior towards a new object. This process depends on the integration of contextual memory with ongoing behavior, and might involve some flexibility in seeking out new objects. While object recognition memory has historically been studied as a HPC-dependent process (Cohen & Stackman, 2015), more recent work has shown that the prelimbic mPFC also contributes to recognition memory. Wang and colleagues (2021) examined the communication between the HPC and mPFC as rats performed the NORT and found that both regions showed increased coupling during novelty discrimination. As such, the NORT may serve as a useful behavioral assay for examining how prefrontal contributions to context-guided behavior might be affected by opioid withdrawal.

Despite the well-established role of the mPFC in cognitive flexibility (Ragozzino, 2007) and the growing evidence implicating it in contextual information processing (de Bruin et al., 1994; Haddon and Killcross, 2011; DeNardo et al., 2019; Samborska et al., 2021), how morphine withdrawal and psilocybin treatment reshape prelimbic neural activity to impact adaptive decision making remains unexamined. Further, it is still unclear how the activity patterns of individual prelimbic neurons evolve across the trajectory of morphine dependence, withdrawal, and recovery in freely behaving rats. The present study addresses this gap by using miniaturized microscopes (Inscopix) in combination with a calcium indicator to record from the same prelimbic neurons across the 18 day experimental paradigm that spans the dependence, withdrawal and recovery phases of morphine use. We also assessed behavioral flexibility throughout the course of this paradigm using a set-shifting task to probe how morphine and psilocybin shape complex goal-directed behavior. Combining this longitudinal approach with the NORT further allows us to ask whether distinct subpopulations of prelimbic neurons are differentially affected by morphine, morphine withdrawal, and whether psilocybin treatment can restore both their neural deficits and the behaviors they support.

Results

To investigate how morphine withdrawal and psilocybin treatment influence flexible and adaptive behavior, and to explore the contributions of prelimbic neurons, we designed an 18-day drug paradigm (Figure 3.1A) with a 2x2 factorial design crossing Drug condition (morphine or saline) with Treatment condition (psilocybin or saline), resulting in four groups (Figure 3.1B): saline–saline (SS), saline–psilocybin (SP), morphine–saline (MS), and morphine–psilocybin (MP). Rats were pseudo-randomly assigned to groups to balance sex across conditions.

Prior to implementing the drug paradigm, rats were trained on a fully automated spatial set-shifting task that required an average of ~18 days (ranging from 8-29 days) to complete. A subset of trained rats underwent surgery for unilateral implantation of an integrated gradient

index (GRIN) lens (Inscopix) above the prelimbic cortex, following infusion of the genetically encoded calcium indicator AAV5.Syn.GCaMP6s.WPRE. These rats were given 4-6 weeks of post-surgical recovery to allow for viral expression prior to beginning the drug paradigm, during which task training was maintained.

Following baseline testing on the day prior to the 18 day drug paradigm (day 0), rats received two daily subcutaneous injections of morphine (10mg/kg) or saline (12 hours apart) from days 1 through 10 to establish dependence. On day 11, injections were withheld to allow morphine rats to enter spontaneous withdrawal. On day 12, rats received a single subcutaneous dose of psilocybin (1 mg/kg) or saline according to their assigned treatment. Days 13 and 14 comprised the novel object recognition test (NORT) familiarity and test phases, and day 17 served as the post-treatment time point. Across the full paradigm, rats continued to perform the spatial set-shifting task every other day, as well as daily on the set condition days to allow us to track flexible decision-making throughout dependence, withdrawal, treatment, and post-treatment (Figure 3.1A,B).

A total of 39 rats contributed data to the experiment, though not every animal participated in every facet of the paradigm due to a combination of training criteria and surgical eligibility (Figure 3.1C). Thirty-two rats (82%) reached behavioral criterion on the spatial set-shifting task and contributed to the set-shifting analyses. Twenty-nine rats (74%) completed the NORT and contributed to the behavioral discrimination analyses. A subset of 16 rats (41%) contributed calcium imaging data during the NORT (NORT+CalIm), and 20 rats (51%) contributed calcium imaging data from the open field condition-day recordings (OF+CalIm). Together, these overlapping datasets allowed us to evaluate how morphine withdrawal and psilocybin treatment shape behavior and prelimbic activity across multiple measures.

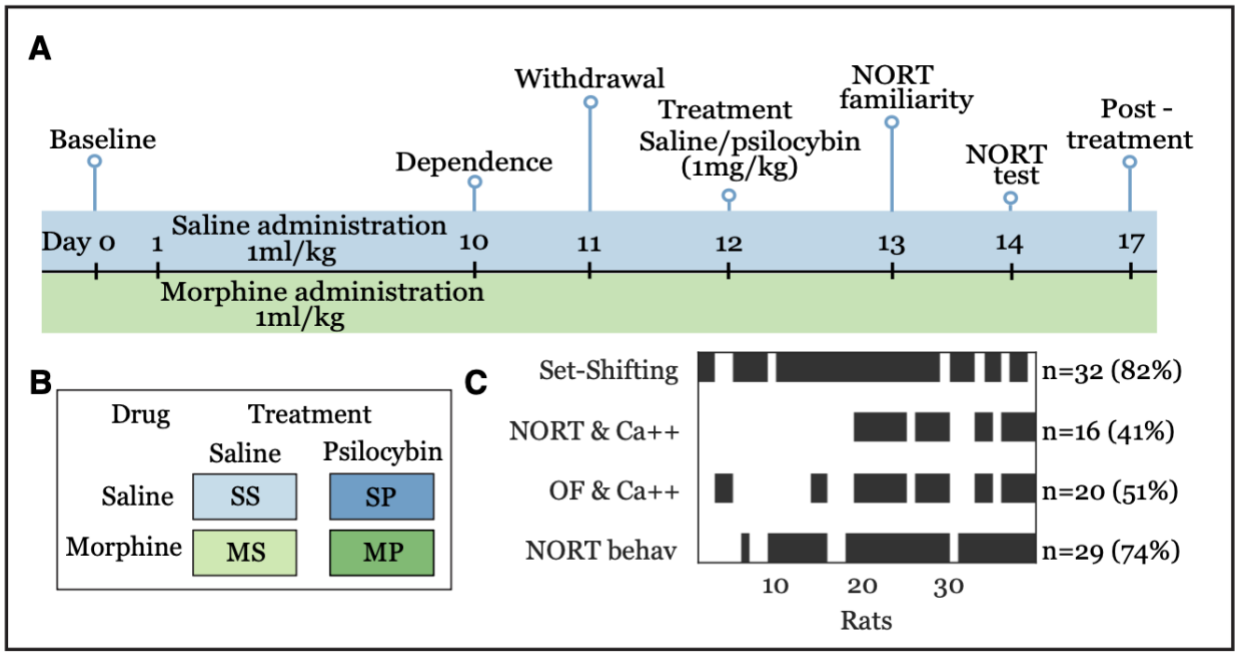


Figure 3.1

Experimental paradigm and rat participation in different phases of the experiment. A) A schematic of the 18-day drug paradigm, with blue denoting the chronic saline-administered group and green denoting the chronic morphine-administered group. B) Rats are a part of one of four groups, resulting from a combination of two Drugs and two Treatments. C) Participation plot of 39 rats included in each part of the experiment.

Confirmation of a morphine-induced maladaptive state and effects of psilocybin

To confirm that our drug administration protocol produced the expected effects on behavior, we first blindly scored two stereotyped behaviors in the open field (Figure 3.2A, top) that served as well- accepted indicators of opiate withdrawal and psychedelic experience in rodents (Halberstadt et al., 2011; Papaleo & Contarino, 2006). Wet dog shakes are a behavioral signature of opiate withdrawal in rodents and are characterized by sudden and large whole-body shakes during active withdrawal. Wet dog shakes were significantly elevated in the rats that were administered morphine, when compared to saline controls (Figure 3.2B; Wilcoxon rank-sum test, $z= -3.641$, $p=0.000$). Head twitches, an indicator of effects of psychedelics (e.g. hallucinations; Erkizia-Santamaria et al., 2025), closely resemble wet dog shakes, though they are less pronounced and more restricted to the shaking of the head. We show that head twitches were also significantly elevated in psilocybin-treated groups, with both SP and MP rats showing more head twitches than their saline-treated counterparts (Figure 3.2C; Wilcoxon rank-sum test, SS vs SP: $z= -2.443$, $p= 0.029$; MS vs MP: $z= -3.072$, $p= 0.004$). These results validated that both morphine and psilocybin had the intended effect on behavior.

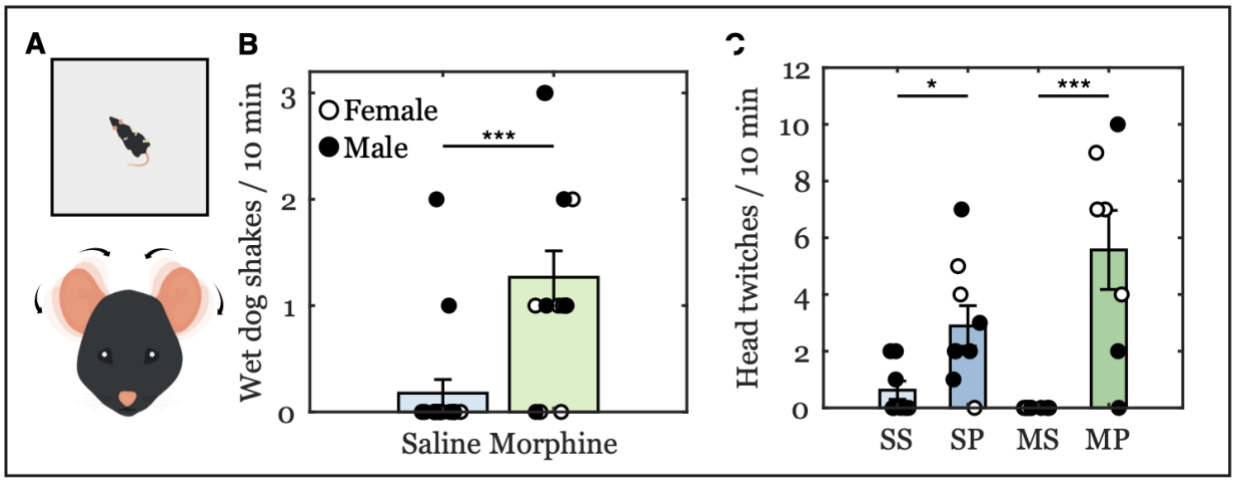


Figure 3.2

Behavioral proxies for morphine withdrawal and psychedelic effect. A) Schematic of the plexiglass open field box (60 cm × 60 cm × 40 cm) and rat head twitch behavior. B) 10 days of twice-daily chronic morphine administrations resulted in significant increases in wet dog shakes, a proxy for morphine withdrawal, relative to chronic saline administrations (Wilcoxon rank-sum test, z= -3.641, p=0.000). C) A single dose administration of psilocybin treatment (1mg/kg) resulted in significant increases in head twitches, a proxy for psychedelic experience in rodents, relative to saline treatment controls in both groups (Wilcoxon rank-sum test, SS vs SP: z= -2.443, p= 0.029; MS vs MP: z= -3.072, p= 0.004).

Morphine impairs strategy switching during dependence & withdrawal

To assess the effects of morphine on behavioral flexibility and adaptability, rats performed a set-shifting task across baseline, dependence, withdrawal, treatment, and post-treatment conditions (see Methods). For each condition, we aligned three trial-level measures across 15 trials immediately before and after 2 key task-relevant events—the block switch (BS), when reward contingencies shifted without cue, and the learning point (LP), the trial at which a rat has adopted the new target strategy. The three trial-level measures were the change in strategy likelihood (CSL; which represents our quantification of flexible responding in terms of the likelihood that rats change task strategy; see Methods), the rate of vicarious trial and errors (VTE; a head-sweeping signature of deliberative behavior), and trial accuracy (Outcome; measured as the proportion of correct responses). We also examined general performance on the task, such as the number of blocks rats were able to complete, CSL following correct and incorrect choice outcomes, and whether different choice outcomes led to increases or decreases in the target strategy use, which would inform the use of adaptive behavior.

The 10th day of morphine or saline administration served as the *Dependence* day. During dependence, rats that were chronically administered morphine completed significantly fewer blocks than saline-administered rats, relative to each rat's own baseline (Figure 3.3A; $t(102)=2.791$, $p=0.006$). This suggested a reduced ability to successfully switch strategies across sessions. Despite this reduction in strategy switching, there was an overall increase in flexibility in morphine rats relative to saline rats (Figure 3.3B; $t(81.79)= -3.735$, $p=0.000$), as CSL was significantly increased following both correct (Figure 3.3C; $t(81.21)= -3.047$, $p=0.003$) and incorrect (Figure 3.3D; $t(87.66)= -3.731$, $p=0.000$) choice outcomes. In support, the trial-aligned analyses showed that morphine rats exhibited greater CSL seven trials before the LP (Figure 3.4A, right; $P=0.021$, $d=2.705$). This was indicative of more willingness to change and

shift strategies throughout the course of the session. Morphine rats also showed an increase in VTE behavior three trials just before and at the block switch (Figure 3.4A, left; P=0.01-0.025, d=2.625-3.099), pointing to greater deliberation or uncertainty when the strategy rules shifted. These results suggest that morphine-dependent rats exhibit greater flexibility and deliberation that coincides with the impaired ability to appropriately switch strategies on the set-shifting task.

On the day following the last morphine injection, Day 11, rats were subjected to spontaneous withdrawal (or the saline-equivalent). As in the dependence day, morphine rats completed significantly fewer blocks than saline rats relative to baseline (Figure 3.3A; t(102)= 2.355, p=0.020), indicating that the deficit in the ability to switch strategies persisted into withdrawal. However, unlike the uniform increases in CSL that morphine rats exhibited during dependence, withdrawal increased CSL selectively following incorrect choice outcomes (Figure 3.3D; t(86.81)= -2.391, p=0.019). This indicates that rats were more likely to change their strategy after making errors than saline rats. Accuracy five (Figure 3.4B, left; P=1, d=-4.231) and six (Figure 3.4B, left; P=0.999, d=-4.280) trials preceding the BS was significantly lower in morphine rats than saline rats, while VTEs were consistently elevated surrounding the LP (Figure 3.4B, right; P=0.007 - 0.014, d=3.190 - 2.868). These results suggest that morphine withdrawal increased flexible responding following negative outcomes, which may mirror the heightened uncertainty/deliberative behaviors rats exhibited as they were adapting to new strategies. This combination may have contributed to the decrease in accuracy prior to reaching the BS, as well as the overall impairment in the number of blocks completed.

On day 12, rats received a single subcutaneous dose of psilocybin or saline as their assigned treatment. About 20 minutes following the treatment, rats were subjected to the set-shifting task. Our comparisons revealed that saline rats treated with psilocybin (SP) showed elevations in CSL 12 (Figure 3.4C, left; P=0.023, d=2.602) and 13 (Figure 3.4C, left; P=0.012, d=2.958) trials following the BS but reduced following the LP (Figure 3.4C, right; P=0.975 -

0.983, d=-2.531 - -2.885), though this averaged to a net decrease in CSL following correct choices (Figure 3.3C; t(80.73)= -2.139, p=0.036). This pattern of results suggests that SP rats were more willing to change their strategy use following the rule switch (exploration) and were also more stable in their strategy use during exploitation. Consistent with this, SP rats also showed reduced VTE behavior both surrounding the BS (Figure 3.4C, left; P=0.98-0.975, d=-1.337 - -2.6163) and 13 trials after the LP (Figure 3.4C, right; P=0.977, d=-2.402), indicating that psilocybin reduced uncertainty along key moments on the task. Both SP (Figure 3.4C, right; P=0.008-0.014, d=1.468-3.216) and MP (Figure 3.4C, right; P=0.015, d=3.274) rats showed elevated trial accuracy following the LP relative to their drug group (SS and MS, respectively), strengthening the use of the target strategy once it has been adopted. Importantly, the effects of psilocybin were present in both saline and morphine groups, indicating that psilocybin's positive effects were not solely restricted to the maladaptive morphine-withdrawn group.

To determine whether the effects of psilocybin persisted five days following treatment, we tested the rats on the set-shifting task once more on day 17. While the morphine rats showed no differences between saline and psilocybin treatments, some of the psilocybin effects in saline rats persisted while others were reversed. For instance, SP rats continued to show reduced VTE rates nine trials prior to the BS (Figure 3.4D, left; P=0.981, d=-2.687) and two trials after the LP (Figure 3.4D, right; P=0.994-0.989, d=-3.207 - -3.002) compared to SS, pointing to a lasting reduction in deliberative behavior at key task events. However, SP rats also showed reduced accuracy three trials following the LP (Figure 3.4D, right; P=0.988, d=-2.372), which is in contrast to the positive effects on accuracy during treatment.

Together, these results show that long-term morphine exposure disrupts set-shifting performance across multiple measures, such that while flexibility increased, uncertainty, accuracy, and the overall ability to complete switches was impaired. A single dose of psilocybin reverses some aspects of these deficits in morphine rats while also improving flexible strategy use in saline control rats.

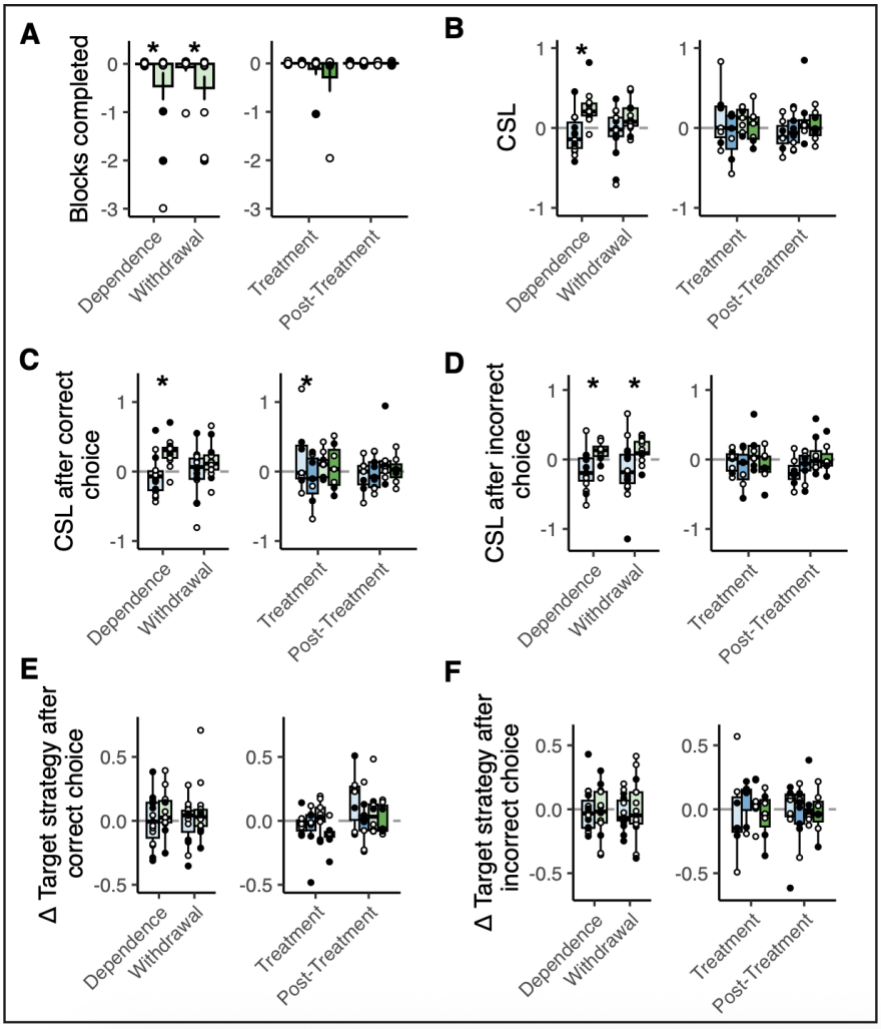


Figure 3.3

Performance on the set-shifting task was impaired during dependence and withdrawal, coinciding with an increase in flexible responding. A) The number of blocks rats were able to complete, was decreased following morphine administration during dependence (t(102)=2.791, p=0.006) and withdrawal (t(102)= 2.355, p=0.020), relative to saline-administered rats. B) The Change in Strategy Likelihood (CSL) was elevated following morphine administration during dependence (t(81.79)= -3.735, p=0.000). C) CSL was elevated following correct choice after chronic morphine administration during dependence (t(81.21)= -3.047, p=0.003), though decreased following psilocybin treatment in the control group (t(80.73)= -2.139, p=0.036). D) CSL was elevated following incorrect choices after chronic morphine administration during dependence (t(87.66)= -3.731, p=0.000) and withdrawal (t(86.81)= -2.391, p=0.019). E) There was no difference in the change in target strategy following correct or incorrect (D) choices. All measures are reported as subtracted values from each rat's baseline performance.

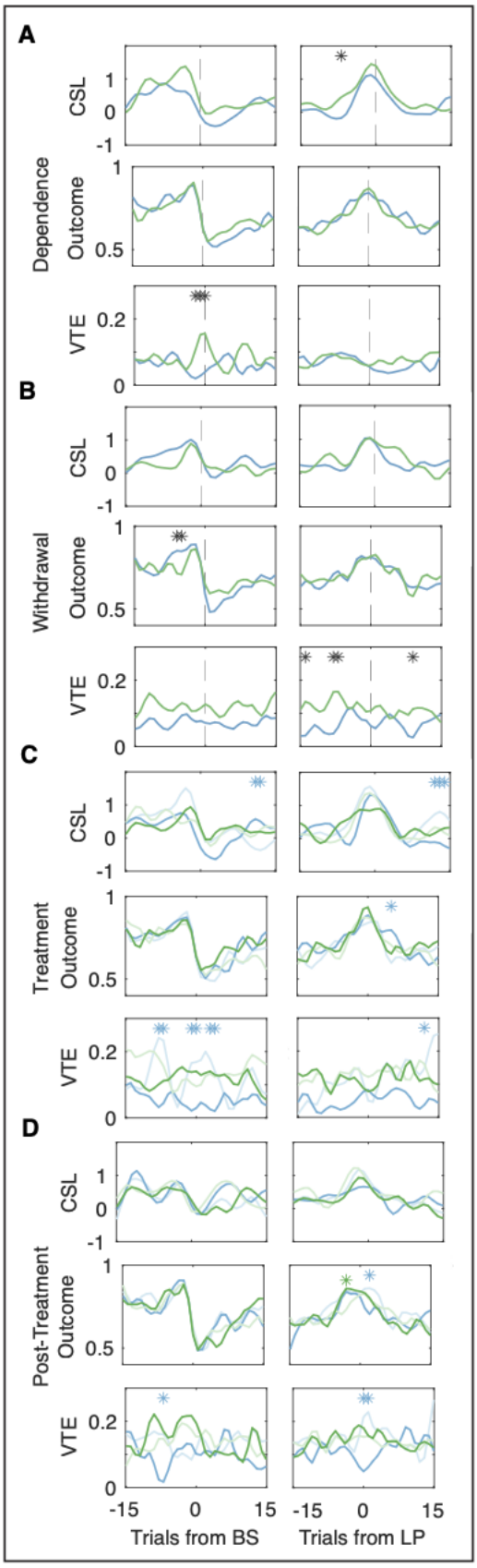


Figure 3.4

Alterations in behavior near key moments on the set-shifting task. A) During dependence, morphine-administered rats exhibited elevations in CSL 7 trials before the learning point (LP; P=0.021, d=2.705), and elevations in Vicarious Trial and Errors (VTE) just before and at the block switch (BS; P=0.01–0.025, d=2.625–3.099). B) During withdrawal, morphine-administered rats exhibited decreases in choice outcomes 5 (P=1, d=-4.231) and 6 trials (P=0.999, d=-4.280) before the BS, alongside elevations in VTEs surrounding the LP (P=0.007–0.014, d=3.190–2.868). C) During treatment, saline rats that were treated with psilocybin, exhibited elevations in CSL 12 (P=0.023, d=2.602) and 13 (P=0.012, d=2.958) trials after the BS, but decreased CSL after the LP (P=0.975–0.983, d=-2.531 to -2.885). Additionally, saline rats treated with psilocybin showed reduced VTE rates surrounding the BS (P=0.98–0.975, d=-1.337 to -2.616) and 13 trials after LP (P=0.977, d=-2.402), coinciding with increases in choice outcomes following the LP (P=0.008–0.014, d=1.468–3.216). Morphine rats treated with psilocybin exhibited increases in

choice outcomes following the LP (P=0.015, d=3.274). Comparisons in C and D were run within each Drug group (ie. SS vs SP and MS vs MP), with blue asterisks representing significant trials between saline groups and green asterisks representing significant trials between morphine groups. Comparisons in A and B were run between saline and morphine rats, with black asterisks denoting significant trials.

Morphine withdrawal impairs novel object discrimination

To probe the impacts of morphine and psilocybin on the contribution of the mPFC to contextual information processing, we combined in vivo calcium imaging of prelimbic neurons with the novel object recognition test (NORT). By the time rats were subjected to the NORT, all four groups had received both the drug (morphine or saline) and the treatment (psilocybin or saline), allowing us to ask how the combination of pre-existing maladaptive state and psychedelic intervention shaped recognition behavior (N = 29; SS: n=8, SP: n=8, MS: n=7, MP: n=6). On day 13, rats explored an open field arena containing two identical smooth objects for 10 minutes (familiarity phase). Twenty-four hours later, we replaced one of the familiar objects with a novel object that differed in shape, color, and texture in the same location. Then we recorded behavior and calcium activity simultaneously across a 10-minute test session (Figure 3.4A).

Recognition behavior was quantified using a discrimination index (DI), calculated as the time spent interacting with the novel object divided by the total interaction time across both objects, with positive values indicating a preference for the novel object and negative values indicating a preference for the familiar object. Across the full 10-minute test session, the overall duration (Figure 3.5B) and total number of interaction bouts (Figure 3.5C) with novel and familiar objects did not differ across groups. Likewise, the DI did not differ significantly across groups (Figure 3.5D; Wilcoxon rank-sum test, $p > 0.05$). However, the MS group was the only group whose mean DI fell below zero, indicating that morphine-withdrawn rats receiving the saline control treatment rats spent more time interacting with the familiar object than the novel one.

Because rats could change their behavior over the course of a test session, and since object recognition behavior may evolve over time, we parsed the NORT test data into two 5-minute time bins. During the first half of the session, all four groups preferred interacting with the novel object, with no significant differences between groups (Figure 3.5E; Wilcoxon rank-

sum test, $p > 0.05$). In the second half, however, the MS group exhibited a shift toward the familiar object, while the MP group continued to preferentially explore the novel object, resulting in a significant difference between the two groups (Figure 3.5E; Wilcoxon rank-sum test, $z= -2.357$, $p = 0.037$). This pattern indicates that morphine-withdrawn rats are specifically impaired at sustaining novel object discrimination across the test session, and that a single dose of psilocybin is sufficient to restore this behavior to control levels.

Additionally, parsing the DI data by sex revealed that the effects of psilocybin on discrimination behavior was driven more strongly by males. MP male rats showed significantly lower DIs than both SS (Figure 3.5D; Wilcoxon rank-sum test, $z=2.087$ $p = 0.037$) and SP groups (Figure 3.5D; Wilcoxon rank-sum test, $z=2.195$ $p = 0.028$), suggesting that males in the MP group were performing significantly poorer than saline controls. In female rats, the opposite pairwise comparison between MS and MP groups trended in the same direction but did not reach significance (Figure 3.5D; Wilcoxon rank-sum test, $z=-1.945$ $p = 0.052$). These results may suggest some sex-differences in the effects of psilocybin in novelty recognition, though this is difficult to interpret with the limited per-group sample size.

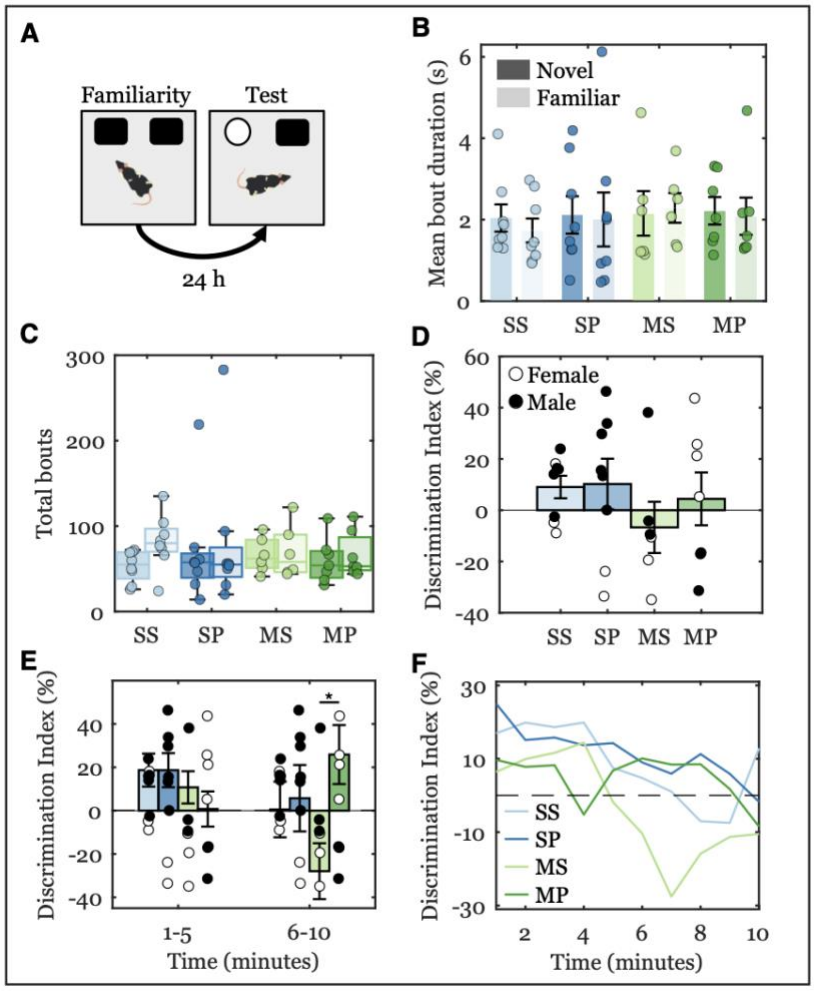


Figure 3.5

Morphine rats treated with saline exhibit impairments in novel object discrimination. A) Schematic of the novel object recognition test (NORT). B) The average bout duration in seconds is consistent across groups and object type. C) The total number of bouts is consistent across groups and object type. D) There were no overall differences in discrimination index (DI) across the groups, however male rats in the MP group exhibited higher DIs than both SS (Wilcoxon rank-sum test, z=2.087 p = 0.037) and SP groups (Wilcoxon rank-sum test, z=2.195 p = 0.028). E) When split by 5-minute bins, MS rats had a lower DI in the latter portion of the test than the MP rats (Wilcoxon rank-sum test, z= -2.357, p = 0.037). F) Visualization of DI minute-by-minute.

Prelimbic neural responses during novel object discrimination

To test whether prelimbic neuron activity reflected changes in NORT behavioral patterns observed across groups, we infused the genetically encoded calcium indicator AAV5.Syn.GCaMP6s.WPRE into the prelimbic cortex and implanted an integrated ProView GRIN lens (0.6 mm × 7 mm; Inscopix) above the injection site in a subset of 16 rats (Figure 3.6A; 9 female, 7 male; n = 4 per group). Following 4–6 weeks of post-surgical recovery to allow for viral expression, rats were subjected to the drug administration paradigm shown in Figure 3A. Calcium activity was recorded simultaneously with behavior throughout the NORT test session. To isolate prelimbic responses to discrete object interactions, we restricted analyses to object interactions separated by at least 2 seconds (Figure 3.6B). We then normalized each cell's trial-averaged response to the median and median absolute deviation (MAD) of that 2-second baseline window (Figure 3.6C).

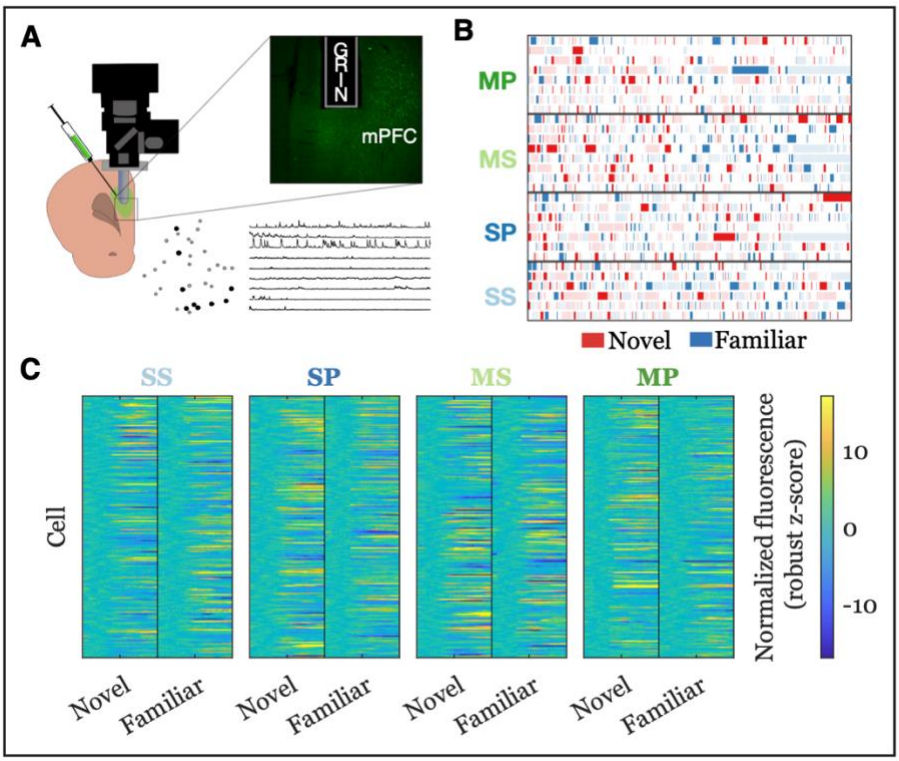


Figure 3.6

Preparing calcium imaging data for analysis. A) On the left, a schematic of a coronal brain slice with a gradient index lens (GRIN) terminating in the prelimbic region of the medial prefrontal cortex (mPFC), with the green calcium indicator, GCaMP6s. To the right, a histological image verifying placement and viral expression, alongside region of interest contours with example trace snippets from select neurons (bolded). B) An ethogram of novel (in red) and familiar (in blue) object interactions across the four groups, with rats on the Y axis and the 10-minute NORT session on the X axis. Faded interactions were less than 2 seconds in proximity and are removed from subsequent analyses. C) Heatmap of peri-event z-scored activations of each cell's responses to novel and familiar objects across the four groups.

To examine group differences in how prelimbic neurons respond to novel and familiar objects, we computed a *neural* discrimination index (Neural Index, NI) for each rat. The NI was defined as the difference in mean post-event activation between novel and familiar object interactions, where the *event* was the onset of object interaction. The calcium activation was then normalized by the total absolute activation, such that positive values indicate stronger prelimbic responses to novel objects and negative values indicate stronger responses to familiar objects. When examining the full 10-minute test session, the NI pattern across the four groups closely paralleled the behavioral DI: MS rats were the only group whose mean NI fell below zero (M=-0.781, SEM=0.541), indicating that prelimbic neurons in morphine-withdrawn rats responded more strongly during familiar object interactions than during novel ones. Pairwise comparisons did not reach statistical significance (Figure 3.7A; Wilcoxon rank-sum, $p > 0.05$).

When the neural data were parsed into the same 5-minute bins used for the behavioral analysis, the NI pattern remained largely consistent across the two halves of the session, with MS rats showing reduced novel object responses in both halves of the session (Figure 3.7B; Wilcoxon rank-sum test, $p > 0.05$). This pattern of neural stability in response to object interactions contrasts with the DI, which diverged between MS and MP only in the second half of the session; the correlation between DI and NI did not reach statistical significance (Figure 3.7C; GLM: $F(1,14) = 1.71$, $p = 0.213$). These results reveal that morphine withdrawal produces a maladaptive neural state in the prelimbic cortex that is present from the start of the NORT, before any behavioral group differences emerge.

To further dissect the source of the MS-MP group difference, we examined event-aligned calcium responses to novel and familiar object interactions within each group. Although prelimbic activity in SS rats was variable and did not reliably distinguish object types, both SP and MP rats showed significant increases in activation aligned to novel object interactions in the seconds following the onset of the interaction (Figure 3.7D, E; SP: 1-2s: Wilcoxon rank-sum test, $z=4.171$ $p = 0.001$; MP 0-3s: Wilcoxon rank-sum test, $z=3.613$-4.508 $p < 0.01$). In contrast, MS

rats were the only group to show significant increases in activation to familiar object interactions one second following the onset of the interaction (Figure 3.7D, E; Wilcoxon rank-sum test, $z=-3.700$ $p = 0.004$). However, 2 seconds prior to the interaction, MS rats exhibited the typical, albeit weak, increase in activation when approaching the novel object (Figure 3.7D, E; Wilcoxon rank-sum test, $z=3.055$ $p = 0.045$). The increase in response to novel object interactions may contribute to the MS group's impaired behavioral discrimination of the novel object.

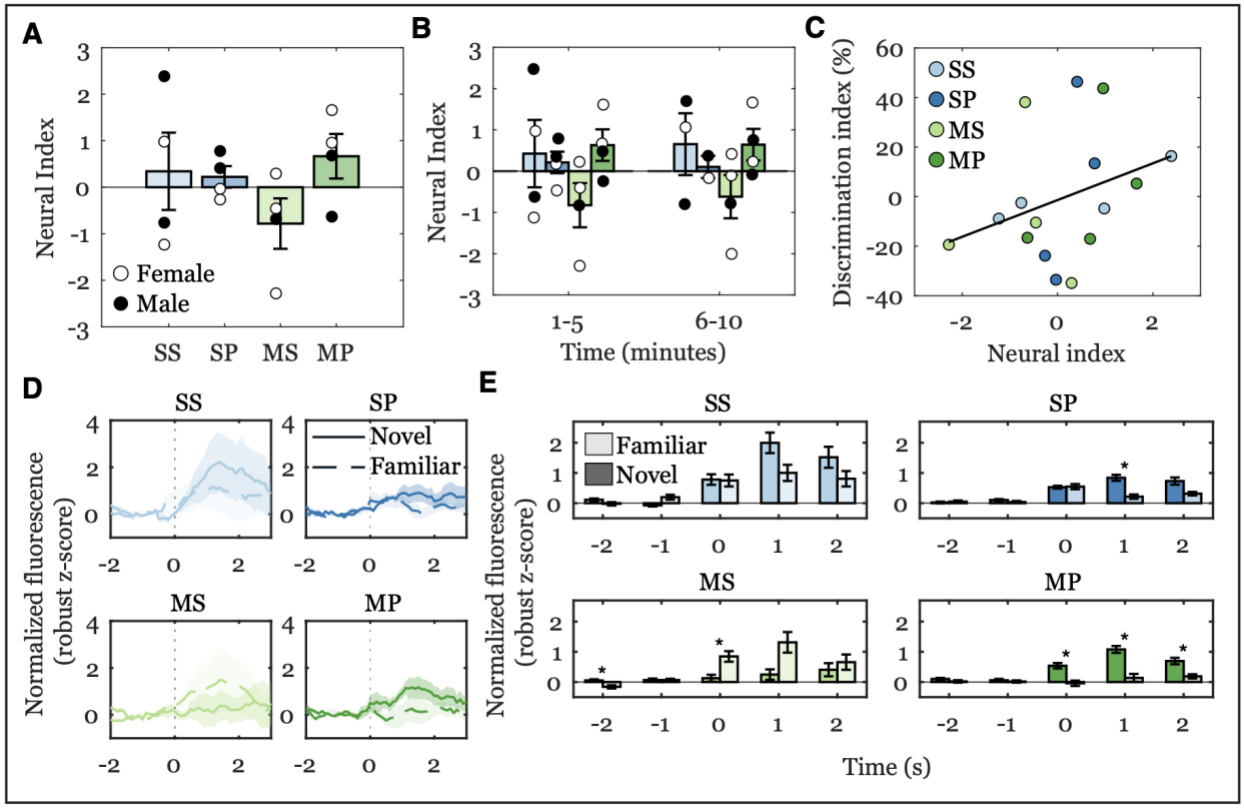


Figure 3.7

Prelimbic activity in response to novel object recognition is influenced by drug and treatment. A) The neural index (NI), representing the prelimbic activation to novel objects subtracted from the activation to familiar objects, was not significantly different across the four groups. B) When the NI was parsed into two 5-minute time bins, there was no difference across the four groups. C) There was a slight positive correlation between the DI and NI, though this did not reach statistical significance (GLM: $F(1,14) = 1.71$, $p = 0.213$). D) The z-scored responses to novel (continuous line) and familiar (dashed line) across the four groups differed. E) Specifically, SP and MP rats exhibited significantly more activation to novel objects when compared to familiar in the seconds following interaction onset (SP: 1-2s: Wilcoxon rank-sum test, $z=4.171$ $p = 0.001$; MP 0-3s: Wilcoxon rank-sum test, $z=3.613$-4.508 $p < 0.01$), whereas MS rats exhibited significantly more activation to the familiar object during interaction onset (Wilcoxon rank-sum test, $z=-3.700$ $p = 0.004$).

Distinct prelimbic subpopulations support novel object discrimination

Given the anatomical and functional heterogeneity of the mPFC (Anastasiades & Carter, 2021), we were interested in further deciphering whether subclasses of prelimbic neurons showed intrinsic patterns of activity. To test whether these subpopulations existed, we used an unsupervised clustering analysis on the pooled peri-event calcium trace matrices of all recorded prelimbic neurons (see Methods). This produced three well-separated clusters (Figure 3.8A).

The three clusters corresponded to distinct response profiles (Figure 3.8B, C). Cluster 1 (n=460 neurons) was selectively activated during familiar object interactions and showed no response to the novel object (Wilcoxon rank-sum test, $z= -20.849$, $p < 0.01$) relative to the pre-interaction baseline. Cluster 2 (n=593 neurons) showed the opposite profile, responding selectively to the novel object (Wilcoxon rank-sum test, $z= 24.244$, $p < 0.01$). Cluster 3 (n=640 neurons) was broadly inhibited during both types of object interactions relative to the pre-interaction baseline, though showed a stronger inhibition during novel object interactions (Wilcoxon rank-sum test, $z= -4.290$, $p < 0.01$). It was of interest to examine whether these subpopulations of prelimbic neurons differentially contributed to the rats' ability to discriminate the novel object. The proportion of neurons in each cluster did not differ significantly across SS, SP, MS, and MP groups (Figure 3.9A; Wilcoxon rank-sum test, $p >0.05$). We also determined whether cluster identity was spatially organized, that is and whether neurons of close spatial proximity were more likely to belong to the same cluster, though this did not appear to be the case (Figure 3.9B).

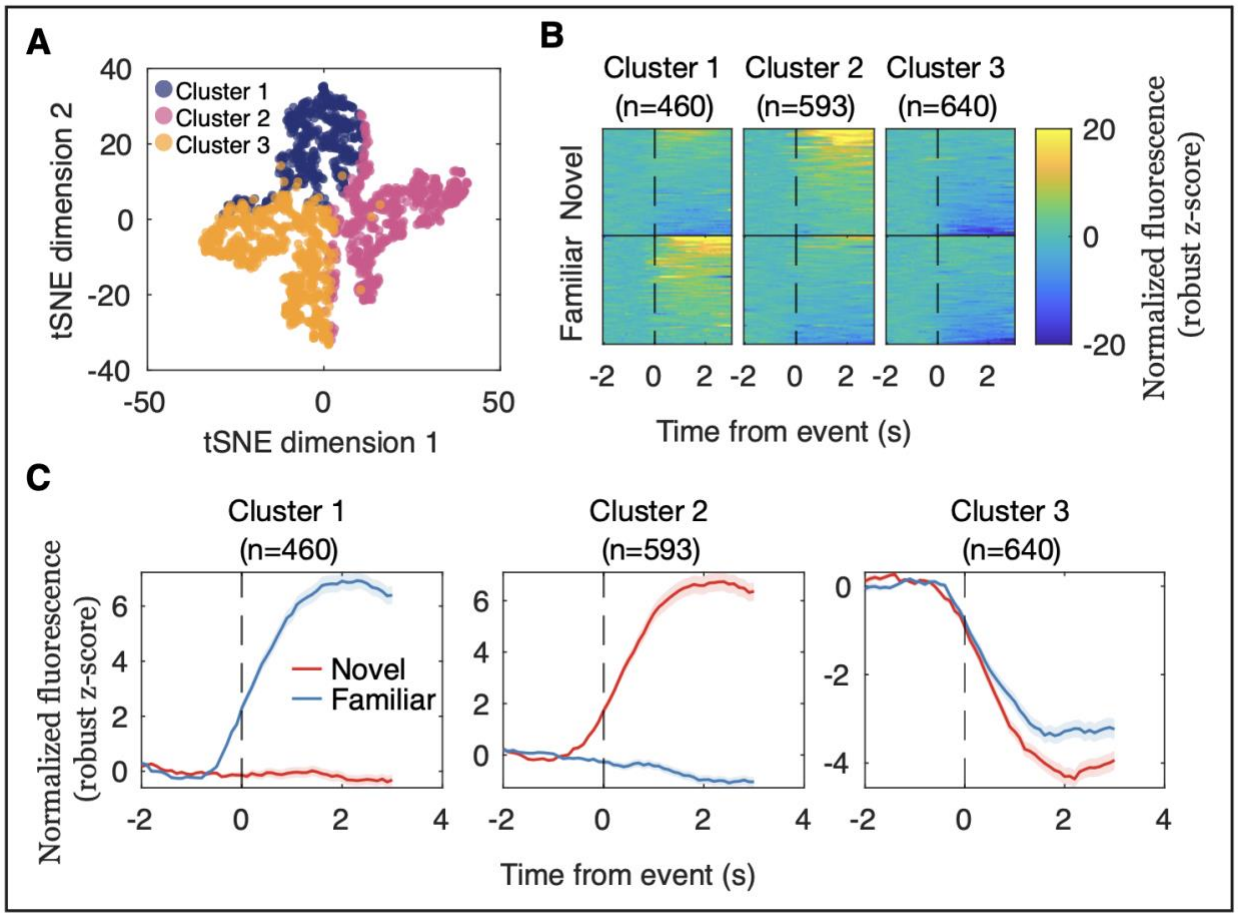


Figure 3.8

Three subpopulations of prelimbic neurons differentially respond to novel and familiar object interactions. A) Neural data was reduced to 2 principal components and projected in 2D space using tSNE. B) Three unique clusters were identified, differing in their response to novel and familiar object interactions. C) Z-scored activity of each of the three clusters is aligned to 2 seconds before and 3 seconds after novel and familiar object interaction onset. The response to novel and familiar objects of each of the clusters was significantly different (Wilcoxon rank-sum test, z= -4.290-24.244, $p < 0.01$).

Although the overall proportion of neurons in each cluster was preserved across groups, the magnitude of within-cluster responses differed between groups. Cluster 3, the broadly inhibited population, exhibited significantly more inhibition in MS rats than MP rats during novel object interactions (Wilcoxon rank-sum test, $z=-2.165$, $p < 0.05$). This suggests that morphine withdrawal suppressed this subpopulation's response during novel object interactions and that psilocybin treatment rescued this effect. For cluster 1, the familiar-selective population, the responses in the MS group trended toward increased activation during novel object interactions compared to MP rats (Wilcoxon rank-sum test, $z= 1.876$, $p=0.061$). No significant within-cluster group differences emerged in cluster 2, however the subpopulation's responses to familiar objects in the MS and MP groups appeared to be less inhibited than the saline groups (SS and SP), suggesting that morphine exposure may influence cluster 2 neurons during familiar object interactions.

Also of interest was whether the proportion of neurons in each cluster could predict an individual rat's neural index (NI) and discrimination index (DI). The proportion of cluster 1 neurons was significantly negatively correlated with a rat's NI ($r=-0.86$, $p=0.000$), such that rats with a higher fraction of familiar-selective cells showed population-level activity tilted toward the familiar object. The proportion of cluster 2 neurons (novel-selective) showed a positive correlation with NI that trended toward significance ($r=0.49$, $p=0.057$), while cluster 3 did not reach significance ($r=0.38$, $p=0.148$). At the behavioral level, however, no cluster proportion significantly predicted DI (cluster 1: $r =-0.31$, $p =0.235$; cluster 2: $r =0.04$, $p =0.874$; cluster 3: $r =0.16$, $p =0.564$). These results show that the distribution of functional subpopulations in the prelimbic cortex supports the neural signature of novel object discrimination but does not map onto the behavioral signature of novel object discrimination directly.

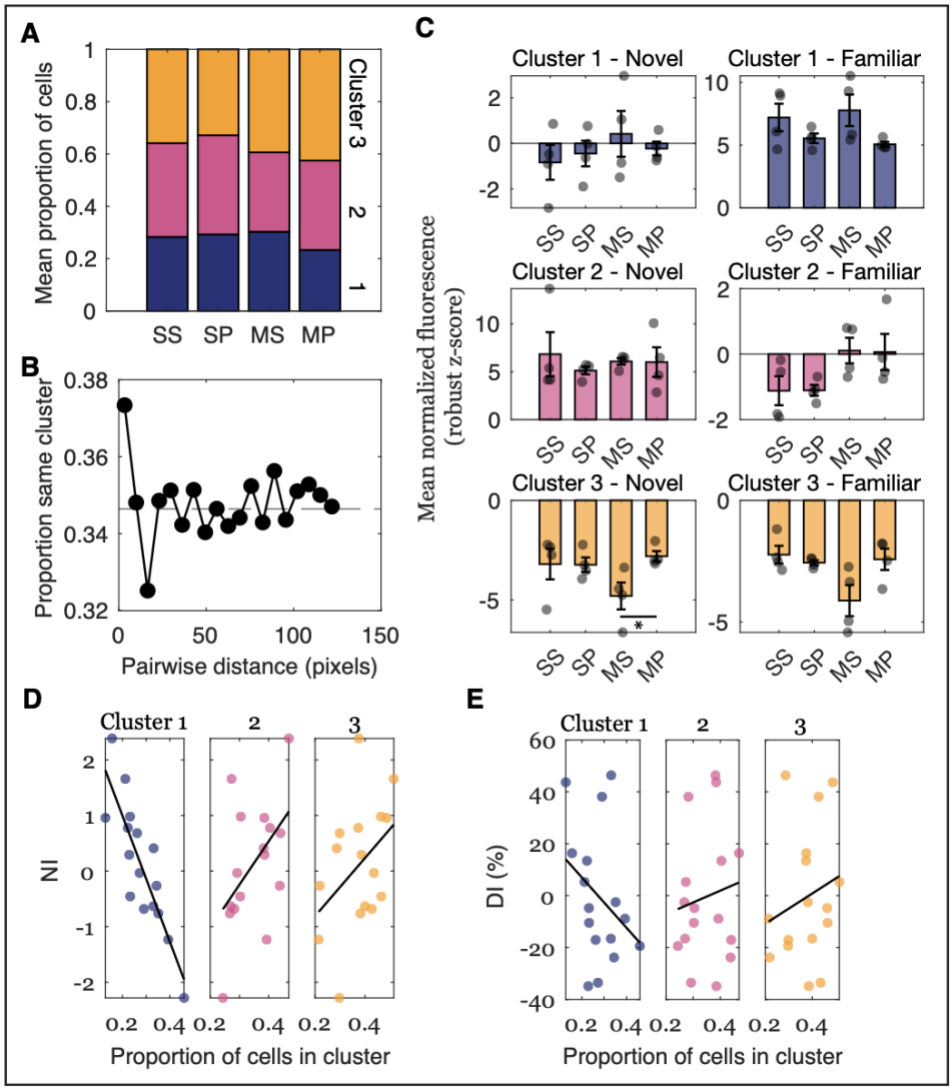


Figure 3.9

The subpopulations within the prelimbic mPFC are differentially affected by drug and treatment. A) The proportion of clusters across the four groups did not differ. B) The spatial proximity of the regions of interest did not influence cluster identity. C) The average z-scored response by cluster and object interaction across the four groups. Cluster 3 of MS rats exhibited more inhibition than MP rats during novel object interactions (Wilcoxon rank-sum test, $z=-2.165$, $p < 0.05$). D) The proportion of cells in Cluster 1 significantly correlated with the NI, while the other clusters did not reach statistical significance (Cluster 1: $r=-0.86$, $p=0.000$; Cluster 2: $r=0.49$, $p=0.057$; Cluster 3: $r=0.38$, $p=0.148$). E) There was, however, no correlation between cluster proportion and DI.

Activity of prelimbic neurons over the course of drug administration varies by drug and treatment

To examine how the activity of prelimbic cells changes across our five drug administration phases and four combinations of drug/treatment groups, we recorded calcium transients on each of the condition days from day 0 to day 17 while rats (n=19) were in an open field box for 10 minutes. We found that there were a comparable number of cells tracked across the conditions and groups (Figure 3.10A). Out of all cells tracked (n=6536), the mean z-scored fluorescence of the MS group, when compared with the MP group, was significantly elevated during withdrawal ($t(377)= 2.179$, $p=0.030$), treatment ($t(421)= 2.648$, $p=0.008$), and post-treatment (Figure 3.10B; $t(293)= 3.098$, $p=0.002$). The MS group, which is the only group to undergo morphine withdrawal without subsequent psilocybin treatment, showed elevated prelimbic activity consistent with previous research showing that mPFC activity is elevated following protracted morphine withdrawal (Piao et al., 2017; Qu et al., 2020); this was not the case for MP. Interestingly, cells in the SP group showed significantly lower activation during withdrawal compared to SS (Figure 3.10B; $t(927)= 2.189$, $p=0.029$). These results suggest that psilocybin may have a general inhibitory effect on prelimbic neurons, which is also supported by previous literature (Celada et al., 2013; Morgan et al., 2023).

Lastly, we tracked the activity of individual cells over the course of the session to see how each cell's activity profile changed in response to the conditions. To reliably track the same neurons over the course of our 18-day experiment, we utilized an astrometry-inspired quad generation algorithm (Stars2Cells) which allowed us to track identical neurons across our 5 sessions consecutively. Out of a total of 6536 tracked cells, 3871 were unique. Although a little less than half of all cells tracked were present in multiple consecutive conditions, about 2% were identified across the entire 18-day experiment (Figure 3.10B). To illustrate how each cell's mean activation across conditions differed by group (SS, SP, MS, MP), a correlation matrix is shown (Figure 3.10D), where each block represents all cells included in both comparison conditions.

The overwhelming majority of cells were uncorrelated across most pairs of conditions. However, there were a couple notable effects. In the SS group, the cells present during baseline and dependence were significantly correlated with one another (Figure 3.10D; r= 0.26, p=0.004, n=125). Likewise, in the MP group, the cells present during baseline and treatment were significantly negatively correlated with one another (Figure 3.10C; r=-0.61, p=0.006, n= 19), potentially owing to the inhibitory effects of psilocybin treatment. In summary, the activity profiles of individual prelimbic neurons are dependent on the combination of drug and treatment, with psilocybin treatment alleviating the hyperexcitability of prelimbic neurons following morphine withdrawal.

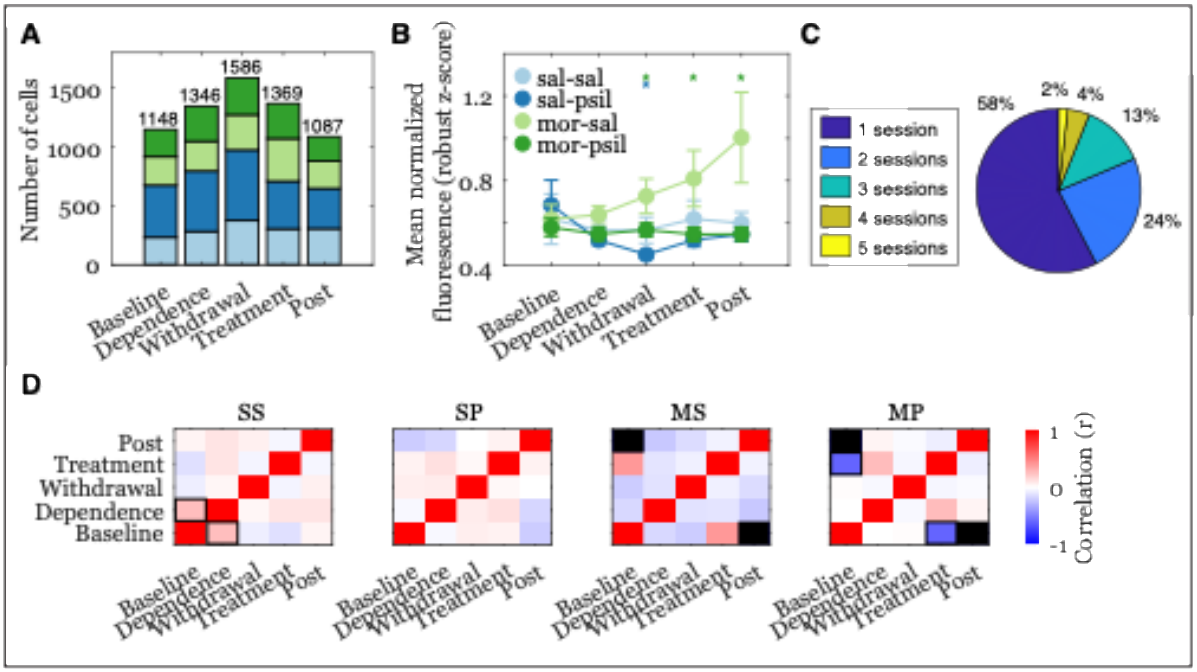


Figure 3.10

The activity of prelimbic cells across the five experimental conditions was influenced by drug and treatment. A) Distribution of the number of total recorded cells (n = 6536) across conditions and groups. B) The mean z-scored activity of cells in morphine rats treated with saline (MS) was significantly greater than those treated with psilocybin (MP) during withdrawal ($t(377)= 2.179$, $p=0.030$), treatment ($t(421)= 2.648$, $p=0.008$), and post-treatment ($t(293)= 3.098$, $p=0.002$) conditions. During withdrawal, saline rats not yet treated with psilocybin exhibited lower activation compared to their saline-treated counterpart ($t(927)= 2.189$, $p=0.029$). C) A pie chart showing the percentage of unique cells tracked across multiple sessions. D) Pairwise Pearson correlations were computed across conditions for cells that were reliably tracked across multiple sessions. Cells within the SS groups exhibited positively correlated activity during Baseline and Dependence ($r= 0.26$, $p=0.004$, $n=125$), while cells within the MP group showed negatively correlated activity during Baseline and Treatment ($r=-0.61$, $p=0.006$, $n= 19$).

Discussion

Opioid use disorder (OUD) is a debilitating condition characterized by impairments in flexible and adaptive behavior and reward valuation that have long-lasting effects. The present study tested the effects of morphine dependence and withdrawal on flexible and adaptive behavior on a spatial set-shifting task. The study also examined how morphine alters the activity of prelimbic neurons in the mPFC during a HPC-dependent task to uncover the putative context-related information processing that the mPFC and HPC might be supporting. Importantly, of interest was whether a single dose of psilocybin could alleviate any of the behavioral and neural effects of withdrawal. We show that morphine dependence and withdrawal impaired multiple aspects of flexible behavior in the set-shifting task, weakened both prelimbic neural and behavioral responses to novel objects in the novel object recognition task (NORT), and resulted in broad hyperactivity in prelimbic neurons that extended beyond the period of morphine exposure. A single dose of psilocybin was sufficient to reverse several of these impairments.

Morphine withdrawal induces a maladaptive, hyperflexible behavioral state

Our 10-day twice-daily administration of morphine, as well as the induction of spontaneous withdrawal 24 hours after the last administration, was sufficient to induce the characteristic symptoms of withdrawal, consistent with other similar paradigms in the literature (Figure 3.2B; Fadaei et al., 2015). Morphine dependence and withdrawal impaired strategy switching on the set-shifting task, as evidenced by a significant reduction in the number of blocks morphine rats were able to complete (Figure 3.3A), as well as decreased choice outcomes and increased uncertainty at key points in the task (Figure 3.4A, B), relative to saline rats. This finding is consistent with earlier literature showing that chronic opioid exposure impairs performance on flexibility-related tasks in both human and animal models (Rapeli et al., 2006; Halbout et al., 2024; Piao et al., 2017). However, the impairment observed in this study

coincided with increases in flexible responding, as revealed by a measure of flexibility, the change in strategy likelihood (CSL) metric (Figure 3.3B, C, D; Figure 3.4A). The paradox may be explained by considering what researchers are referring to when they discuss flexibility. In much of the literature, flexibility is inferred from the outcomes of flexibility-dependent tasks. This includes the number of reversals, perseverative errors, or trials to criterion, which reflect whether an animal has adapted to changes in task contingencies. However, in these flexibility-dependent tasks, stability is necessary to demonstrate adaptation. For instance, once a new rule has been learned, it is important to maintain use of that rule until the rule shifts again. Our CSL metric measures flexibility more specifically by quantifying the trial-to-trial change in strategy use irrespective of whether that change leads to a successful outcome. By our measure, rats exposed to chronic morphine were not inflexible as previous literature may suggest, but instead were significantly more flexible than saline controls, which resulted in impairments on the task. Their behavioral impairment is therefore better characterized as maladaptive hyperflexibility.

During morphine withdrawal, the maladaptive hyperflexibility was demonstrated following negative choice outcomes (Figure 3.3D), where morphine-withdrawn rats showed elevated CSL values specifically after incorrect trials, and not correct trials (Figure 3.3C). Consistent with this, Verharen and colleagues (2020) showed that the prelimbic area of the mPFC processes negative feedback, whereby inactivating the prelimbic area resulted in deficits in punishment learning. A study by Evers and colleagues (2005) showed that tryptophan depletion in human subjects, which depletes serotonin levels, impaired their ability to update and adjust behavior on a set-shifting task despite negative feedback. Given that morphine withdrawal similarly depletes serotonin levels in the mPFC (Goeldner et al., 2011), the maladaptive hyperflexibility we observed following errors may reflect a disruption of serotonergic modulation of contextual information about task contingencies, resulting in the decreased ability to complete blocks.

The effects of psilocybin treatment on the set-shifting task were largely isolated to the saline control rats rather than the rats that were chronically administered morphine. During the treatment condition, saline rats treated with psilocybin (SP) showed a significant reduction in CSL following correct trial outcomes (Figure 3.3C), compared to saline rats treated with saline (SS). This suggests that the treatment of psilocybin resulted in increased stability in strategy use following positive feedback, such that they were less likely to change their strategy use. Likewise, CSL after treatment was increased in SP rats immediately following the block switch (BS; Figure 3.4C), and reduced after the learning point (LP; Figure 3.4C). Although too much behavioral stability can be considered too rigid and maladaptive, in this case, SP rats performed better and had better accuracy following the LP than their SS counterparts (Figure 3.4C). More importantly, the pattern of increased CSL at the BS and decreased CSL at the LP is consistent with optimal behavior where rats are readily changing their behavior following a contingency shift, and remaining stable in their strategy use during exploitation. For an insightful review on the effects of psilocybin on stability and flexibility, see Sayal & Barrett (2023). SP rats also exhibited decreased uncertainty, as their VTE rates were diminished surrounding the BS and after the LP (Figure 3.4C).

During the five-day post-treatment phase of the experiment, some of psilocybin's effects maintained, while others did not. SP rats continued to show reduced VTE rates relative to SS rats (Figure 3.4D), suggesting a lasting reduction in uncertainty on the task. However, we saw decreased accuracy after the LP, suggesting that rats had more difficulty exploiting the adopted strategy (Figure 3.4D). On the other hand, MP rats showed increased accuracy in the trials immediately preceding the LP (Figure 3.4D), relative to MS rats, suggesting that the positive effects of psilocybin on rats chronically exposed to morphine may develop over time.

Morphine dependence and withdrawal produced clear impairments in strategy switching and behavioral stability. While subsequent psilocybin treatment had a number of positive effects

on set-shifting task performance for saline controls, psilocybin had limited effects on morphine-exposed rats during performance of the same task.

Prelimbic contributions to contextual processing are disrupted by morphine withdrawal and rescued by psilocybin

The mPFC has an established role in novel object memory (Tanimizu et al., 2018) that is dependent on an animal's ability to remember familiar objects and seek novelty. Studies have shown a functional, context-based relationship between the hippocampus and the mPFC, such that inactivating the hippocampal inputs to the mPFC results in impaired novel object discrimination (Wang et al., 2021). Given that the hippocampus encodes contextual features of an environment (Eichenbaum, 2007), the mPFC's contribution to novel object recognition likely depends on its ability to receive and integrate contextual information to guide behavior toward a novel object (Tanimizu et al., 2018). In our study, novel object discrimination was tested following our drug and treatment administrations. Morphine-withdrawn rats treated with saline (MS), and not morphine-withdrawn rats treated with psilocybin (MP) or saline controls (SS, SP), were the only group to show impaired novel object discrimination, which was especially apparent in the second half of the novel object recognition test (Figure 3.5 D, E). Although the reduction in the neural index (NI) in MS rats did not reach statistical significance (Figure 3.7A, B), they were the only group to show significantly more activation in response to familiar object interactions relative to the novel object (Figure 3.7D, E). This combination of behavioral and neural effects resembles that of directly inactivating the hippocampal inputs to the mPFC (Wang et al., 2021), suggesting that morphine withdrawal was sufficient to alter prelimbic activity to a point where it no longer may have been able to effectively utilize the contextual information it received from the hippocampus, resulting in increased activation to a familiar object instead of the novel. In support of the theory implicating the hippocampus in mPFC disruption during novel object discrimination, Sánchez-Bellot and colleagues (2022) show two parallel

hippocampal-mPFC pathways that mediate approach and avoidance behavior, and which may be affected during maladaptive states. In addition to the event-specific prelimbic increases during familiar object interactions, we also saw general elevations in the average activity of prelimbic cells in the MS group (Figure 3.10B). This is consistent with previous research that has shown elevations in mPFC local field potential power following morphine withdrawal (Mohammadzadeh et al., 2022). A separate study has shown that morphine diminishes inhibitory transmission from parvalbumin interneurons onto pyramidal neurons in the prelimbic area of the mPFC through mu-opioid receptors, while simultaneously enhancing somatostatin interneuron inhibition of parvalbumin interneurons (Jiang et al., 2021). This results in a two-part disinhibition of pyramidal activity, which may explain the prelimbic hyperactivity following morphine withdrawal.

Importantly, in the case of increased mPFC activation in response to the familiar object instead of the novel (Figure 3.7D, E), as well as for the general elevations in mPFC activation that we saw across the course of the experiment (Figure 3.10B), a single dose of psilocybin was sufficient in reversing these elevations. Although we administered a subcutaneous injection, this rescue effect is likely related to psilocybin's action on the serotonergic system within the mPFC specifically. Serotonin acts primarily as an inhibitory modulator in the mPFC, suppressing glutamatergic activity while simultaneously increasing the activity of inhibitory interneurons (Zhong & Yan, 2011; Kjaerby et al., 2016). Chronic morphine exposure has been shown to deplete serotonin levels in the mPFC (Goeldner et al., 2011), which may have functionally disinhibited the activity in prelimbic cells, resulting in the observed hyperactivity in our morphine-withdrawn rats. Consistent with this theory, Bekinschtein and colleagues (2013) showed that blocking the serotonergic receptors that psilocin, the active metabolite of psilocybin, binds to in the mPFC impaired object recognition memory. As such, in our study, morphine-withdrawn rats (MS) may have exhibited depleted levels of serotonin in the mPFC,

thereby removing the inhibitory modulation of prelimbic cells and resulted in hyperactivity, which in turn disrupted the processing necessary for novelty discrimination.

We identified three functionally distinct populations of prelimbic neurons via a clustering analysis (Figure 3.8). These three distinct populations differentially contributed to novel object discrimination: roughly a third of the recorded cells were familiar-selective (Cluster 1), where the cells were significantly more activated by interactions with a familiar object. Another third were novel-selective (Cluster 2), where cells were significantly more activated by interactions with a novel object. The last third of cells recorded were broadly inhibited by all object interactions (Cluster 3). The proportion of these cells did not differ across groups (Figure 3.9A), suggesting that morphine administration or psilocybin treatment did not shift the overall distribution of prelimbic response types. However, the magnitude of within-cluster responses did differ between the groups (Figure 3.9C). We found that Cluster 3, the broadly inhibited cluster, was significantly more inhibited in its response to novel object interactions in MS rats relative to MP rats that exhibited inhibition levels similar to SS and SP rats (Figure 3.9C). Although not significant, Cluster 3 neuron responses in MS rats in response to familiar objects were also inhibited more than other groups. Additionally, Cluster 2 neurons in morphine rats (both MS and MP) were a lot less inhibited during familiar object interactions, relative to saline rats. These within-cluster differences suggest that morphine exposure selectively reshapes the response profiles of specific prelimbic subpopulations rather than a uniform shift across all cells. Importantly, psilocybin appeared to reverse the morphine withdrawal inhibition in Cluster 3, indicating that a single serotonergic intervention was sufficient to normalize the activity in that subpopulation. However, the reduced inhibition of Cluster 2 neurons during familiar object interactions in morphine-exposed rats, regardless of whether they received psilocybin or saline as treatment, suggests that some morphine-induced changes in prelimbic subpopulations may be more resistant to serotonergic treatment.

These subpopulation-specific effects are consistent with the previous literature showing that the prelimbic area of the mPFC consists of functionally distinct subpopulations that are differentially engaged by drugs and behavioral states. Clarke and colleagues (2024) find that prelimbic neurons projecting to the nucleus accumbens are differentially affected during heroin self-administration. Specifically, the activation or inhibition of these excitatory ensembles increased or decreased the rates of relapse, respectively. Interestingly, the mPFC and nucleus accumbens are two of the most structurally affected regions following drug exposure (Robinson et al., 2002), with the nucleus accumbens also exhibiting significant plasticity effects following treatment with a serotonergic agonist (Ball et al., 2009). Similarly, Muir and colleagues (2024) show that an administration of the psychedelic DOI resulted in varying responses across the recorded prelimbic cells. When these researchers tagged and stimulated the cells that were activated by DOI, mice exhibited anxiolytic effects. Therefore, our findings support the extant literature that prelimbic subpopulations are differentially affected by morphine withdrawal and that psilocybin selectively rescues the activity of specific subpopulations which may contribute to maladaptive and therapeutic states.

The present study aimed to characterize the effects of morphine administration and withdrawal on prelimbic activity, flexible behavior and context processing, and to determine whether a single dose of psilocybin could alleviate the behavioral and neural consequences of withdrawal. Our approach of testing flexible responding on a complex set-shifting task, as well as in vivo calcium imaging during novel object recognition, allowed us to assess the behavioral consequence of morphine withdrawal and the underlying prelimbic effects that accompany them. The results show that morphine withdrawal produced a maladaptive hyperflexible state on the set-shifting task, impaired novel object discrimination, drove hyperactivity in prelimbic cells, and selectively reshaped the response profiles of certain prelimbic subpopulations. A single dose of psilocybin was sufficient to normalize prelimbic hyperactivity and restore behavioral discrimination during novel object discrimination. These findings add to the growing

body of literature implicating the mPFC in the behavioral dysfunction of opioid withdrawal and the therapeutic potential and mechanisms of psychedelics.

Materials & Methods

Subjects

All procedures were conducted in compliance with National Institutes of Health guidelines and received approval from the University of Washington's Institutional Animal Care and Use Committee. Thirty-nine Long-Evans rats (female = 20, male = 19; 3-months old; 250–350g; Charles River Laboratories) were used in this study. Rats were single-housed under controlled temperature conditions on a 12-hour light/dark schedule, and all training and testing occurred during the light phase. In the days leading up to training, each rat was handled for at least 10 minutes daily across five consecutive days. Rats were then placed on a food-restricted diet, maintained at 85% of their ad libitum body weight throughout the duration of training and experimentation.

Apparatus & Training Procedures

A fully automated spatial set-shifting task, originally developed by a previous graduate student in the laboratory (Miles et al., 2024), was used to assess cognitive flexibility. The task was administered on an elevated plexiglass plus maze (arm dimensions: 58 cm long × 5.5 cm wide; elevated 80 cm above the floor), enclosed by black curtains affixed with distinct spatial cues within a sound-attenuated testing room. A custom LabVIEW interface (2016; National Instruments, Austin, TX) operated in a neighboring room allowed experimenters to observe and control the session remotely. An Arduino, integrated with the LabVIEW program, tracked each rat's location via a SONY USB camera (~35 fps) and controlled the motors on the maze arms to raise and lower the arms surrounding the center platform. The ends of the East and West arms had 3-D printed reward ports, while the North and South arms served as pseudo-randomly alternating start positions to discourage egocentric navigation strategies. After retrieving a

reward (sucrose pellet; 45 mg; TestDiet, Richmond, IN, USA) at the East or West arm, rats returned to the North or South arm, which triggered the arms to lower and the rats were restricted to the new start arm for a variable delay of two to five seconds before the next trial initiated and arms raised. On every trial, both reward arms remained accessible.

Each session comprised four counterbalanced allocentric spatial strategy blocks: two place strategy blocks (place-East and place-West) and two Alternation blocks, arranged such that the Alternation blocks were never sequenced consecutively. During place blocks, rats were required to select the same reward arm (East or West, depending on the block) on every trial. During Alternation blocks, rats had to switch between the East and West reward arms on successive trials. The active strategy was never explicitly cued; instead, rats inferred the current rule through trial-and-error based on reward feedback. Block transitions occurred once the rat achieved 12 correct responses within a rolling window of 15 trials, at which point the reward contingencies shifted to the next block without any cue. The task concluded when rats completed 4 blocks or 3 strategy switches, or ran for a maximum of 90 minutes.

To quantify how rats used each of the three available strategies, we computed strategy likelihood estimates using a recency-weighted Bayesian inference procedure described in Maggi et al. (2024) and Miles et al. (2024). For each trial, the success or failure of every possible strategy was evaluated based on the rat's choice (e.g. a choice of a West arm registered as a success for the place-West strategy and a failure for the place-East strategy), and these outcomes were recency-weighted to result in likelihoods of using each of the three strategies (place-West, place-East, and Alternation). This produced a continuous trial-by-trial estimate of the rat's underlying strategy use, allowing us to track how strategies evolved over the course of a session. Two key trial-level events were extracted from each block to characterize how rats responded to changes in reward contingencies. The block switch (BS) represents the trial at which the strategy rule shifts (uncued) and the reward contingencies update, while the learning point (LP) denotes the first trial when the likelihood of the block's target strategy becomes the most likely strategy.

Trials falling between the BS and the LP were labeled as the *exploration* phase, depicting the period of behavioral adaptation following an uncued switch, during which rats are actively sampling possible strategies to identify the new rule. Trials falling between the LP and the BS represents the *exploitation* phase, as rats adapt and use the new target strategy. Together, the BS and LP segment distinct aspects of flexible behavior, allowing us to ask how morphine, withdrawal, and psilocybin shape strategy use.

Thirty-two (16 females) of the 39 rats were first habituated to the maze across two to three days, each consisting of 10 minutes of free exploration and scattered sucrose pellet rewards on the maze. Next, a forced-choice pre-training phase introduced rats to each of the three strategy types (place-East, place-West, Alternation) across separate blocks, in which only one reward arm was available per trial. Sucrose pellet rewards were delivered at the end of each forced trial. Block lengths were gradually increased from 5 to 15 trials per block as performance improved. Once pre-training was complete, rats transitioned to the free-choice version of the task in which both arms were accessible on every trial and blocks were presented in a randomized, counterbalanced sequence. Rats advanced to surgery after successfully completing three strategy switches (four total blocks) within a single session on two consecutive days.

For the Novel Object Recognition Test and Open Field recordings, an open field arena (60 cm × 60 cm × 40 cm) made of plexiglass was used. The floor of the arena was affixed with a matte black vinyl adhesive for grip as well as to prevent reflection during video recordings from the camera above (Basler acA800-510um; Basler AG, Ahrensburg, Germany).

Surgery

Upon reaching set-shifting task criterion, food restriction was lifted and rats were returned to *ad libitum* feeding for one to four days before surgery. On the day of surgery, anesthesia was induced using 4% isoflurane delivered in oxygen (1 L/min flow rate) within an induction chamber. Rats were then secured in a stereotaxic frame (David Kopf Instruments,

Tujunga, CA, USA) and isoflurane was induced between 1% and 3% to sustain an appropriate level of anesthesia.

A subset of the cohort (n = 19; 11 females) was implanted with an integrated gradient index (GRIN) lens to enable chronic calcium imaging of the right hemisphere of the prelimbic cortex. A craniotomy was performed over the target site (AP: +3.00 mm, ML: +0.65 mm, referenced to Bregma), and an infusion needle was slowly lowered to the deepest target coordinate (DV: -4.1 mm from the skull surface). After a 10-minute settling period, 700 nl of AAV5.Syn.GCaMP6s.WPRE (provided by the Zweifel laboratory, University of Washington) was delivered at 60 nl/min. The needle was then raised to -3.7 mm DV, where an additional 700 nl was infused at the same rate, followed by another 10-minute waiting period to limit backflow along the needle tract. An integrated ProView GRIN lens (0.6 mm diameter × 7.0 mm length; Inscopix, Mountain View, CA, USA) was subsequently lowered at a rate of 100 μm/min to -3.9 mm DV at the same AP and ML coordinates. Four stainless steel anchor screws were positioned bilaterally (ML: ±2.5 mm; AP: -2.0 mm and -5.0 mm) and the entire assembly was secured with a layer of C&B Metabond and dental cement.

Rats were given seven days of post-operative recovery with unrestricted access to food and water. Following recovery, food restriction was reinstated to 85% of each rat's free-feeding weight and rats were retrained on the set-shifting task. The experimental paradigm began 4–6 weeks after surgery, providing sufficient time for viral expression of the calcium indicator.

Experimental Design

The experiment spanned 18 days (Day 0–Day 17) and followed a 2 × 2 between-subjects factorial design crossing Drug condition (Saline vs. Morphine) with Treatment condition (Saline vs. Psilocybin). This resulted in four groups: Saline–Saline (SS), Saline–Psilocybin (SP), Morphine–Saline (MS), and Morphine–Psilocybin (MP). Group assignments were pseudo-randomized with sex as a balancing factor.

Day 0 served as the baseline, during which rats performed the set-shifting task (n=32) and, for lens-implanted rats (n=20; 10 females), a calcium imaging session was recorded in the open field. From Day 1 through Day 10, rats received twice-daily subcutaneous injections of morphine or saline (10mg/kg at 1 ml/kg per injection, for a total of 20mg/kg per day) and were subjected to the task every other day to maintain performance; a calcium recording was collected in the open field on Day 10 to capture the dependence condition. On Day 11, injections were withheld, rats performed the task under acute, spontaneous withdrawal (or the equivalent time point for saline-treated rats), and another calcium recording was obtained. On Day 12, a single subcutaneous injection of psilocybin (1 mg/kg) or saline was administered, followed by a 10-minute waiting period in their home cage. Treatment-condition calcium recordings were conducted after the waiting period, immediately followed by the set-shifting task. The novel object recognition test (described below) was performed on Days 13 and 14, with calcium recordings (n=16; 9 females) occurring on day 14 to measure neural responses during the test phase. Day 17 consisted of the post-treatment condition, representing the five days following saline or psilocybin treatment, which included the set-shitting task and a final calcium recording in the open field.

Novel Object Recognition Test

Recognition memory was assessed using a two-day novel object recognition paradigm conducted in an open field arena (60 cm × 60 cm × 40 cm). During the familiarity phase (Day 13), two identical smooth glass box objects were positioned in the arena and rats (n=29; 15 females) explored freely for 10 minutes. Twenty-four hours later, during the test phase (Day 14), one of the previously encountered objects was swapped for a novel object, a textured, striped ceramic container, and rats were again given 10 minutes to explore. For rats with GRIN lens implants (n=16), calcium imaging was performed concurrently during the test phase (Day 14).

In Vivo Calcium Imaging Acquisition

Calcium fluorescence in prelimbic neurons was captured using the nVista miniature microscope (Inscopix, Mountain View, CA, USA) in the 19 lens-implanted rats. Recordings were acquired at 20 Hz for 10-minute sessions across five key experimental time points: baseline (Day 0), dependence (Day 10), withdrawal (Day 11), treatment (Day 12), and post-treatment (Day 17). For each of these sessions, rats explored the empty open field arena, and no concurrent behavioral video was collected. The same field of view was maintained across all recording sessions for each animal, and a subset of the total recorded neurons were tracked across multiple conditions. Lastly, an additional calcium recording was made during the NORT test (Day 14) session.

Temporal synchronization between neural and behavioral data streams was achieved via a TTL pulse emitted from the Inscopix SYNC output port. This pulse triggered the start of video acquisition on a Basler acA800-510um camera (Basler AG, Ahrensburg, Germany) running at 100 fps under the Pylon software suite. Once triggered, the Basler camera collected frames at its native rate, with the initial sync pulse serving as the common temporal reference for offline alignment of the two data streams.

Calcium Imaging Data Processing

All calcium imaging videos were preprocessed in Inscopix Data Processing Software (IDPS; Inscopix). Each video was first cropped to isolate the active field of view, then spatially downsampled by a factor of 4 and temporally downsampled by a factor of 2, reducing the acquisition rate to 10 Hz. A spatial bandpass filter (default IDPS parameters) was applied to attenuate low- and high-frequency spatial noise. Frame-to-frame motion artifacts were corrected by running the motion correction algorithm two to three times, with each pass using a hand-traced region of interest encompassing the full field of view.

Neuronal sources were extracted using the CNMFe algorithm (Zhou et al., 2018) within IDPS. Each putative ROI was then reviewed individually by the experimenter in IDPS's single-cell inspection mode, where the spatial location and temporal trace were evaluated to confirm that the ROI represented a true neuronal signal.

Calcium Signal Normalization & Analysis

For the condition-day recordings, extracted fluorescence traces from the five condition-day sessions were standardized via robust z-scoring, implemented in MATLAB (version 2023b) using the normalize function with the 'zscore' and 'robust' options. This transformation expresses each value as the number of median absolute deviations from the column median, which is resistance to extreme fluorescence transients.

For the NORT peri-event traces, calcium traces recorded during the NORT were segmented around individual object interaction onsets (see Position Tracking & Behavioral Classification), extracting a −2 s to +3 s peri-event window for every qualifying bout. Only bouts separated by at least 2 seconds were retained to minimize temporal overlap. For each neuron, the raw peri-event traces were first averaged across all bouts for a given object type. The trial-averaged trace was then normalized using a robust baseline correction: the median and median absolute deviation (MAD) of the 2-second pre-interaction epoch served as the centering and scaling parameters, respectively.

To determine whether prelimbic neurons separated into distinct and intrinsic response subpopulations, the normalized peri-event traces were analyzed using a clustering pipeline developed by zhounapeuw and colleagues (2023). Briefly, dimensionality was first reduced via PCA, retaining 2 components that captured most of the variance in the population response matrix. Then, the number of clusters and nearest-neighbor values were systematically varied, fitting spectral clustering and k-means models at each combination of values and using the silhouette score to identify the best-fitting combination. The spectral clustering solution with 3 clusters and 28 nearest neighbors achieved the highest average silhouette score (0.64) and was

selected for downstream analysis. Group-level differences in cluster-specific neural responses were evaluated by computing the mean post-interaction activity for each rat within each cluster and object type (novel vs. familiar). Pairwise comparisons across all six group pairs (SS vs. SP, SS vs. MS, SS vs. MP, SP vs. MS, SP vs. MP, MS vs. MP) were performed using Mann–Whitney U tests.

Position Tracking & Behavioral Classification

Markerless pose estimation was performed with DeepLabCut (version 2.2; Mathis et al., 2018), using task-specific models for each behavioral context (set-shifting task and open field). For the plus maze, a model trained to track the head was used (NVIDIA GEFORCE GTX 1080 GPU; 500,000 training iterations), following procedures detailed in Miles et al., 2024. A separate model was trained for the open field NORT, estimating the position of eight body parts (nose, left ear, right ear, left flank, back, right flank, tail base, and tail tip). Object interactions during the NORT were detected using Simple Behavioral Analysis (SimBA; Goodwin et al., 2024), which identified frames in which the nose marker entered a set region of interest drawn around each object in the arena.

Analyses & Statistics

A neural index (NI) was calculated for each animal to quantify the degree to which prelimbic population activity differentiated between novel and familiar objects. NI values were averaged at the rat level and partitioned into 5-minute time bins. Within each bin, two planned comparisons (SS versus SP and MS versus MP) were carried out using Wilcoxon rank sum test. A bonferroni correction was used when appropriate, and significance was set at $p < 0.05$. Correlations were measured using Pearson correlation coefficient, with significance set at $p < 0.05$.

Behavioral performance on the set-shifting task, as well as comparisons of cell activation across conditions, were analyzed using linear mixed-effects models implemented in R (lme4

package; version 2023.09.0) or MatLAB (fitlme package; version 2023b). The model included experimental Condition and Group (SS, SP, MS, MP) as fixed effects along with their interaction term, and individual rat as a random intercept to accommodate the repeated-measures structure. Planned contrasts were evaluated via estimated marginal means (emmeans package) and included a main effect comparison of Saline versus Morphine drug conditions collapsed across treatment, within-drug treatment effects (SP vs. SS; MP vs. MS), and cross-group comparisons (SS vs. MS, SP vs. MP, SS vs. MP, SP vs. MS). A bonferroni correction was used when appropriate, and significance was evaluated at $p < 0.05$.

To quantify trial-to-trial behaviors, behavioral measures, such as Outcome, VTE rates, and CSL, were aligned to 15 trials before and after two key moments in the task: the learning point and block switch. Behavioral patterns were estimated using a bootstrapping procedure outlined in chapter 3. Briefly, a hierarchical bootstrapping procedure was applied at the subject level (resampling rats with replacement within each condition) and block level (N=2 for block switches; N=3 for learning points) for 1,000 iterations. Due to our between-subjects design, condition means were computed separately at each interaction and subtracted, resulting in a condition-level difference. P values were calculated as the proportion of bootstrap differences that fell above or below zero on each trial, while d values represented the standard deviation from the mean. Significance was evaluated at P above 0.975 or below 0.025 combined with a d value magnitude of 1.5 or greater.

Histology

At the end of the experiment, rats were placed under deep anesthesia with 4% isoflurane and euthanized via intraperitoneal injection of sodium pentobarbital (1–2 mL/kg). Rats were transcardially perfused first with 150 mL of phosphate buffered saline and then with 150 mL of 4% paraformaldehyde. Brains were removed, fixed, and coronally sectioned at 40 μm on a

vibratome. Tissue sections were mounted on slides and examined under a fluorescence microscope to verify the position of the GRIN lens tract and the extent of GCaMP6s expression.

Chapter 4

The flexibility continuum

Disruption of the medial prefrontal cortex or lateral habenula results in maladaptive hyperflexibility

As researchers, we naturally focus on the circuits that are most relevant to the questions we are asking. This has to do in part because the more we understand about each individual component, the more the broader picture comes together. In our series of experiments, we focused on the hippocampal – medial prefrontal cortical – lateral habenular circuit (HPC, mPFC, LHb) and its contributions to flexible and adaptive behavior. Flexible behavior is a broad term that encompasses several simultaneous processes that enable us to either remain stable in our strategic choices and behavior or allow us to change our behavior and utilize other alternative strategies. One feature that is necessary for adaptive flexible behavior is contextual information. Knowing where you are, what has happened in similar environments in the past, what choices were made and the outcomes following those choices are all influential in decision-making. The HPC is a structure that encodes this contextual information and represents the spatial and temporal features of an environment in a way that can be retrieved when an animal encounters a similar situation in the future (Vasudevan et al., 2024). Environments are rarely entirely novel, and in most cases share features with a previous context. In this way, the HPC builds and maintains a cognitive map that connects the current environment to previous experiences to inform appropriate choices.

For the contextual information to influence decision-making, it has to reach the structures that guide action selection. The HPC is one of the largest inputs into the prelimbic region of the mPFC (Hoover & Vertes, 2007), which may contribute to the role the mPFC plays in contextually-guided action (Gilmartin & Helmstetter, 2010; Capuzzo & Floresco, 2020).

Specifically, the HPC organizes and maintains contextual cues from past experiences, which is then retrieved by the mPFC to inform the process of determining subsequent behavior (Eichenbaum, 2017). This theory is reflected in our results where we found that inducing a maladaptive state of morphine withdrawal in Long-Evans rats resulted in hyperactivity in the mPFC (Figure 3.10B) which coincided with impaired novel object discrimination (Figure 3.5E). Importantly, while saline controls exhibited greater activation in response to novel objects when compared to familiar object interactions, the morphine group that was exhibiting withdrawal symptoms showed increased activation for the familiar object (Figure 3.7E). Interestingly, a single treatment of psilocybin was effective at both reversing the prelimbic hyperactivity (Figure 3.10B) as well as the novel object discrimination (Figure 3.5E) and response to novel objects (Figure 3.7E). These direct context-related impairments were also somewhat reflected when rats performed on the complex flexibility-dependent set-shifting task (Figure 3.3; Figure 3.4). Notably, the impairments on the task coincided with increased flexibility and deliberative behavior. Thus, the induction of a maladaptive state was sufficient to disrupt mPFC activity, resulting in putative mPFC-related impairments in context retrieval, which manifested as maladaptive hyperflexibility.

Similarly, when we inactivated the LHb, we observed a similar behavioral pattern. Rats exhibited elevated flexibility on the set-shifting task, increased deliberation, and an inability to use negative choice outcomes to inform future choice behavior (Figure 2.2, 2.3, 2.4). Given that the mPFC projects to the LHb directly (Kim & Lee, 2012), and that the LHb itself is necessary for context-related processing (Goutagny et al., 2013), we hypothesize that the LHb receives contextual information that was retrieved by the mPFC and integrates it with motivational and internal state signals to then send to downstream structures (Figure 1.2). Thus, inactivating the LHb resulted in the inability to integrate contextual information and impaired the ability to gauge whether a change in a behavioral strategy was needed. Consistent with this hypothesis, both morphine-induced disruption of the mPFC and direct inactivation of the LHb produce the

same maladaptive hyperflexibility. In other words, disrupting one end of the circuit results in the degradation of the contextual signal before it reaches the LHb (i.e. morphine-induced impairment of the mPFC), while on the other end integration is impaired entirely (i.e. inactivation of the LHb). The result is the same: the circuit loses its ability to appropriately evaluate whether a given strategy should be maintained or changed.

The HPC-mPFC-LHb circuit we have presented here, is of course, one of many that contribute to flexible and adaptive behavior. For instance, the orbitofrontal cortex and the dorsomedial striatum has been implicated in mediating flexible choice behavior on spatial tasks (Braun & Hauber, 2011; Ragozzino, 2007). Likewise, the medial septum (Bortz et al., 2022), as well as the pathway between the mPFC and medial septum are necessary for strategy switching (Bortz et al., 2023). The ventral tegmental area is another structure that, like the mPFC, has subpopulations within it that track contextual cues and contingency reversals (Lefner & Moghaddam, 2025). Thus, it is very likely that the maladaptive hyperflexibility we observed reflects not only the dysfunction of the HPC-mPFC-LHb circuit alone, but also the downstream effects on the broader network of structures that collectively support flexible behavior.

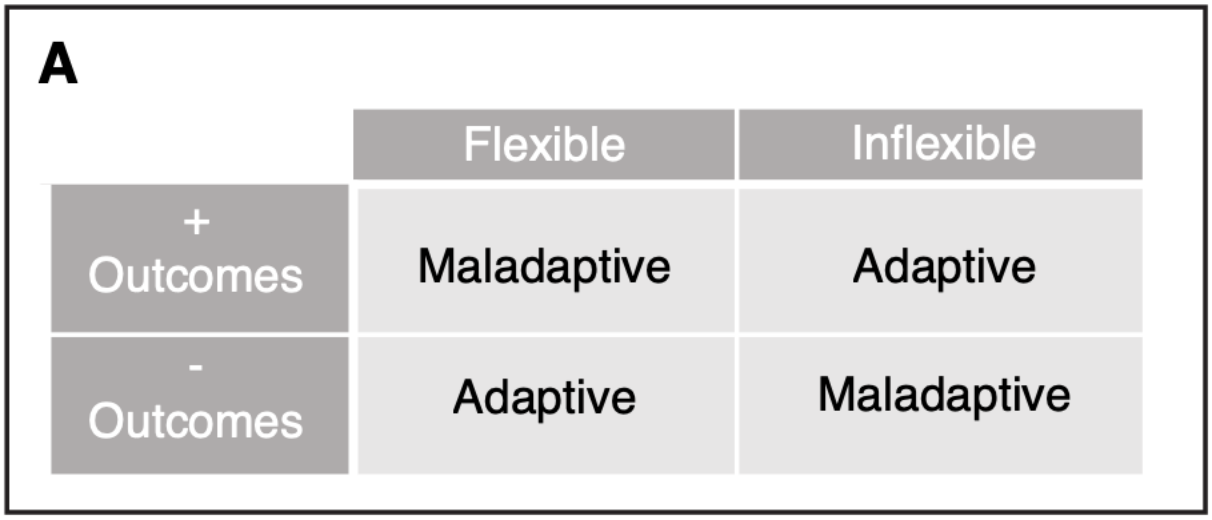


Figure 4.1

A schematic describing the flexibility spectrum. A) A combination of outcome and flexible or inflexible responding can lead to either adaptive or maladaptive behavior. For example, when a behavior has resulted in a positive outcome, it would not be beneficial to change that behavior (i.e. respond flexibly), therefore leading to a maladaptive response. This diagram is specific to behaviors on tasks in the laboratory.

To be or not to be…flexible

Flexibility, i.e. shifting a given cognitive or behavioral pattern, is not inherently good, and stability, i.e. maintaining a given cognitive or behavioral pattern, is not inherently bad. Both are features of adaptive behavior that must be employed at the right time and the right context. After a negative outcome, increased flexibility is adaptive so that an animal might change its behavior and try something different. After a positive outcome, stability is adaptive where an animal should maintain the strategy that is working. Flexibility and rigidity are therefore fluid functions that shift in response to the demands of the situation, and it is the context that determines whether behavior is adaptive or maladaptive (Figure 4.1). When an animal is flexible when they should be stable, or stable when they should be flexible, pathologies characteristic of psychiatric conditions may emerge.

We generally characterize depression as a disorder of rigidity and hyperstability (Stange et al., 2017). Individuals with depression have difficulty changing their routines, become resistant to new experiences, and ruminate on negative thoughts. Thus, depression is a pathology of inflexibility (or hypoflexibility) where the individual struggles to shift out of maladaptive patterns even when there is a need to be flexible. Inflexibility has also been linked to obsessive-compulsive disorder (Frota Lisboa Pereira de Souza et al., 2024), autism spectrum disorder (Ferretti et al., 2024), and generalized anxiety disorder (Ottaviani et al., 2016). As such, disorders characterized by a lack of flexibility, such as depression, have been subject to behavioral interventions in an effort to decrease depressive symptoms by way of increasing flexibility. One such behavioral intervention is cognitive restructuring, where patients are guided and encouraged to challenge their own beliefs and develop alternative behavioral responses (Santos et al., 2024). Consequently, cognitive restructuring has shown to improve depressive symptoms, self-esteem, and decrease stress levels (Santos et al., 2024).

What is, then, the pathology of hyperflexibility? Our findings suggest that morphine withdrawal is characterized by a hyperflexible state that results in some maladaptive

performance outcomes. When we induced morphine withdrawal in rats, they became hyperflexible and shifted their behavioral patterns at elevated rates. This coincided with increased deliberative uncertainty, as well as hyperactivity in the mPFC. As such, we have shown that morphine withdrawal, specifically in rats, is characterized as a disorder of maladaptive hyperflexibility. Similarly, attention deficit hyperactivity disorder (ADHD) is characterized by attentional instability and impulsivity, which reflects difficulty remaining stable. Interestingly, roughly 21% of patients treated for substance use disorder also meet the criteria for ADHD (Rohner et al., 2023). The fact that there is a comorbidity between the two disorders suggests that there may be a common underlying functional impairment. Similarly to the behavioral interventions used for depression, inhibitory control training has proven an effective tool in improving emotional regulation in children diagnosed with ADHD (Groves et al., 2022). Although the findings here are the first to characterize morphine withdrawal as a disorder of hyperflexibiliy, there have been some behavioral interventions that have attempted to target the *increased impulsivity* symptoms of substance use disorder with response inhibition training which have proven to be efficacious (Verdejo-Garcia, 2016).

This collection of studies highlights the importance of looking at specific behavioral patterns of psychiatric pathologies more closely when determining the behavioral intervention. Rather than assuming that all pathologies are characterized by rigidity and would benefit from increased flexible responding, we should instead be looking more closely at the behavioral patterns to determine whether or not an increase in flexibility per se would be beneficial.

The inferential trap

The Chinese Room by John Searle (1980) illustrates a fundamental problem with inferring processes from outcomes. In the thought experiment, a person who doesn't speak Chinese is locked in a room with a rulebook that specifies which Chinese characters to write in response to any series of Chinese characters they receive. Outside of the room, native Chinese

speakers slide messages under the door without the knowledge that there is anyone in the room, and the person in the room must use the rulebook to respond with a series of Chinese characters. From the outside, it appears that *the room* understands Chinese. However, the person inside does not understand the characters they are receiving or responding with and is simply following a set of instructions. The outcome, a coherent response of Chinese characters, would be the same if the room *did* understand Chinese. However, clearly the process leading to the outcome can vary.

Such a functionalist approach of relying on outcome measurements only can lead to incomplete and even incorrect interpretations of data in scientific research. As such, in studies where researchers present animals with a task that depends on flexibility, and only the outcome (e.g. choice accuracy) is measured, it might be difficult to infer whether flexibility was increased or decreased. Specifically, poor performance on a flexibility-dependent task does not necessarily equate to inflexibility. Unless we look *inside the room*, that is we directly measure flexible behavior, there is little that we can infer about the process of flexibility itself. In our experiments, we directly measure flexibility, the process of maintaining or changing different strategies by generating trial to trial strategy likelihood estimates. This allowed us to discover that, although the outcome of inactivating the LHb as well as inducing a maladaptive state that impaired mPFC activity resulted in poor performance on our flexibility-dependent set-shifting task, the process of flexible responding that led to these maladaptive decisions was actually elevated.

This difference in measuring process and outcome is not unique to the study of flexibility. Berridge and colleagues (2009) showed that dopamine, which has been associated with mediating pleasure, is involved in wanting rather than liking. That is, animals will pursue rewards they don't enjoy. The behavioral output looks the same, but the underlying process is different. Likewise, when we measure perseveration on a strategy-dependent task, are we actually looking at animals making choices consistent with the previous strategy, or are we

simply counting errors? If we are only counting errors (i.e. the outcome), it is possible that an animal is using another incorrect strategy instead of perseverating on the previous strategy. Our trial-to-trial strategy likelihood estimates allow us to discern and measure the difference between hyperflexibility and hypoflexibility, as well as whether an animal is perseverating on a previous strategy directly, which would not have been possible when looking at the outcome measures alone. As our methods and tools become more precise, so will our ability to better infer the processes that drive behavior.

From rats to humans

Animal models are useful in developing methods for the treatment of various psychiatric disorders, as well as for our basic understanding of the circuits that underlie certain behaviors. The strength of animal research is the ability to isolate, manipulate, and record from specific structures, which is often not possible in humans. As such, in our series of experiments, we inactivated one node of the circuit (LHb) to examine the effects on flexibility, as well as induced a maladaptive state (morphine withdrawal) and recorded calcium activity from another node (mPFC). This allowed us to characterize the contributions of each node of the circuit to flexible behavior. However, animal models necessarily simplify the conditions that are studied. Specifically in our study, forced morphine administration does not capture the self-administration, escalation, or relapse cycles that is characteristic of human addiction. Withdrawal symptoms alone do not precipitate an addiction in animals (Stewart & Wise, 1992). What our model does allow for is the isolation of effects of chronic opioid exposure and spontaneous withdrawal on the mPFC, which we can link to flexible responding.

The HPC-mPFC-LHb circuit has clear homologs in the primate brain, both functionally and anatomically (Kawai et al., 2015; Rudebeck & Izquierdo, 2022), and the manipulation of homologous structures across species tend to show similar effects (Luigjes et al., 2012). In addition, the complex set-shifting task that we used is analogous to the human test of cognitive

flexibility such as the Wisconsin Card Sorting Task. Thus, although we show the contribution of the circuit to maladaptive hyperflexibility, morphine withdrawal, as well as the successful reversal of neural and behavioral impairments following psilocybin treatment in rats, further research is necessary to confirm these effects in other animal models and clinical settings.

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