Doctoral theses at NTNU, 2024:17

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Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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Kavli Institute for Systems Neuroscience

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ISBN 978-82-326-7636-1 (printed ver.)
ISBN 978-82-326-7635-4 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

Doctoral theses at NTNU, 2024:17

Printed by NTNU Grafisk senter

Contents

Norwegian summary

Medial prefrontal cortex (mPFC) har blitt studert omfattende på tvers av ulike arter, antatte funksjoner og eksperimentelle paradigmer. Til tross for dette har vi fortsatt ikke fått oversikt over nøyaktig hva dette hjerneområdet faktisk gjør, og hvordan disse funksjonene er representert samtidig i mPFC. Spesielt ikke i naturalistiske, frie omgivelser. Det er konsensus i feltet om at det mediale prefrontale området er viktig for fleksibel atferd og kontekstuelt passende valg av handlinger. For at et hjerneområde skal kunne utføre de nødvendige beregninger for disse handlingene så må et bredt spekter av ulike kognitive funksjoner utnyttes og ulike informasjonsstrømmer på tvers av modaliteter integreres inn i en helhetlig representasjon av den aktuelle konteksten man befinner seg i. Dette inkluderer også representasjonen av pågående atferder hvor nødvendige justeringer kan bli gjort basert på sensorisk feedback og forandringer i ens interne tilstand. Arbeidet i denne avhandlingen reflekterer en innsats for å forstå hvordan mPFC er i en posisjon til å lykkes med alle disse komplekse beregningene samtidig. Med den hensikten gir arbeidet her innsikt i potensielle anatomiske baner basert på arbeid i mus, demonstrerer et at- ferdsbasert paradigme som er avhengig av slike anatomiske baner, samt viser at den faktiske nevrale representasjonen av atferd forandrer seg dynamisk i rotter som beveger seg fritt.

En visuomotorisk bane som er viktig for adekvat interaksjon med objekter i ens omgivelser er kjent som den dorsale strømmen for visuell prosessering. Dette konseptet introduseres i min første studie ettersom arbeidet jeg presenterer her karakteriserer denne banen i mus mer detaljert enn tidligere studier. Denne studien undersøkte konnektiviteten og overlappet mellom primær visuell cortex (V1) sine efferente fibre og nevroner som projiserer til sekundære motoriske cortex (M2) i extrastriate cortex i mus. Ved å analysere detaljerte 3D-rekonstruerte celler bekreftet vi at visuelle og motoriske baner overlapper i extrastriate områder, hovedsakelig i anterior og mediale extrastriate områder. Dette bekrefter tidligere funn av en slik bane mellom visuelle og motoriske områder. Ettersom disse områdene projiserer til blant annet prefrontale områder så skaper dette en anatomisk basis for å undersøke hvordan prefrontal cortex benytter seg av visuomotorisk informasjon for å informere kognitive prosesser, for eksempel ved sosiale interaksjoner, inkludert under observasjonell læring.

Man hypotiserer at prefrontale områder spiller en rolle i observasjonell læring basert på at de er innblandet i en rekke ulike aspekter av sosialkognisjon i tillegg til deres omfattende koblinger med sensoriske, motoriske og kognitive områder.

Min andre studie hadde som hensikt å studere observasjonell læring uten å måtte basere seg på bruk av frykt eller noen former for deprivasjon for at dyrene skulle være motiverte for å lære. Dette forskningsfeltet mangler et paradigme som kan muliggjøre undersøkelser av andre hjernebaner enn cortico-limbiske baner, eller for å muliggjøre undersøkelser av selve prosessen for observasjonell læring i et dyr som ikke er stresset. I mitt arbeid presenterer jeg et nytt eksperimentelt paradigme for observasjonell læring med rotter, som ikke baserer seg på frykt eller deprivasjon som motivasjon for læring. Ved bruk av intrakraniell stimulering er dyret som observerer i stand til å lære seg en atferdssekvens med 2 steg etter bare tre dager, kun ved å observere veltrente demonstratører. Når disse observatørene ble testet presterte de bedre enn kontrolldyr, noe som indikerte at de hadde lært via observasjon. En subgruppe av observatørene viste seg derimot å ikke ha lært atferdssekvensen. På grunn av dette foreslår jeg framtidige justeringer av protokollen i håp om at mitt arbeid har lagt grobunn for ett solid observasjonelt læringsparadigme som ikke baserer seg på frykt.

Ettersom mPFC har gjentatte ganger vist seg å være involvert i sosialkognisjon i tillegg til mange andre atferdsmessig relevante funksjoner så benyttet min tredje studie seg av en upartisk tilnærming for å analysere nevral data i rotter som beveger seg fritt i ulike atferdskontekster. Ettersom de fleste studier av prefrontale områder har blitt utført i strengt kontrollerte eksperimenter med fokus på ulike isolerte kognitive aspekter så ville jeg bryte med tradisjonen og karakterisere den faktiske nevrale dynamikken i mPFC under naturalistisk atferd, som kunne gi en helhetlig forklaring på de ulike funnene i litteraturen. For å oppnå dette ønsket jeg at dyr skulle få bevege seg fritt og oppføre seg naturlig i ulike kontekster hvor de kunne bli ledet til å utvise ulike naturalistiske og spontane atferder, slik dyr gjør utenfor en eksperimentell situasjon. Med dette klarte jeg å vise at mPFC har nevrale representasjoner på populasjonsnivå for ulike atferdstilstander som utvikler seg dynamisk basert på hvordan dyret interagerer med sine omgivelser. Potensielt rekrutteres enkeltnevroner inn i betydningsfulle ensembler basert på dyrets momentære behov og motivasjon. Individuelle fysiske trekk var ikke tilstrekkelig for å forklare helheten av atferdsrepresentasjonene, noe som indikerer at dyrets interne tilstand, kognitive prosesser og/eller sensorisk input fra omgivelsene kan bidra til disse representasjonene. Med dette viser jeg at mPFC koder fleksible atferder som er nødvendig for å løse økologiske relevante oppgaver, og jeg bruker denne studien som en bakgrunn for å diskutere hvor viktig det er å benytte seg av både velkontrollerte og naturalistiske eksperimenter for å få en omfattende forståelse for hjernens funksjon.

English summary

The medial prefrontal cortex (mPFC) has been studied extensively across species, across functions and across experimental paradigms. Despite this, we still have not fully elucidated what this brain area truly does and how, especially in naturalistic, non-restrained environments. There is consensus in the field that the medial prefrontal area is important for behavioral flexibility and contextually appropriate action selection. For a brain area to successfully perform these kinds of computations, a vast range of cognitive functions must be utilized and different sensory information streams must be integrated into a coherent representation of the current context. This includes representations of ongoing behaviors, so that necessary adjustments can be made based on sensory feedback and changes in one's internal state. The work in this thesis reflects an effort to understand how the mPFC is able to integrate these disparate computations successfully. In doing so, the work here gives insights into a possible anatomical pathway by which visual signals reach frontal cortices in mice, demonstrates a social learning paradigm that likely depends on such a pathway, as well as showing that the actual neural representation of behavior dynamically changes in freely moving rats.

A visuomotor pathway important for enabling proper interaction with objects in one's spatial surroundings is known as the "dorsal stream" of visual processing. This concept is introduced in the first paper, and the work I present characterizes anatomical connections in this pathway in mice in greater detail than previous studies. The study investigated the intersection of primary visual cortex (V1) output fibers onto secondary motor cortex (M2)-projecting neurons in the extrastriate cortex of mice. Utilizing high resolution analyses on 3D reconstructed cellular data, we confirmed that visual and motor pathways overlap in extrastriate cortex, primarily in the anterior and medial extrastriate areas. This confirms previous findings of such a pathway between visual and motor areas. As these areas also project to other prefrontal areas, this pathway constitutes a possible anatomical pathway supporting visuomotor behavior and may inform cognitive processes, like during social interactions, including observational learning. The prefrontal areas are hypothesized to be important for observational

learning based on their involvement in various aspects of social cognition, as well as their extensive connections with sensory, motor, and cognitive areas.

My second study investigated observational learning, for which I developed a paradigm that does not rely on fear, aversive stimuli or food deprivation to motivate the learning of a task. The field has been lacking such a paradigm that would enable mechanistic investigations of pathways other than cortico-limbic ones in social fear learning, or how the process of observational learning is represented in the brain when animals are not under stress. In the paradigm, using intracranial stimulation as a reinforcer, observer animals are able to learn a 2-step behavioral sequence purely through observation of well-trained demonstrators after only three days of observation. The observer animals outperformed control animals during testing, indicating successful observational learning. However, a subgroup of animals did not learn the task. Hence, I suggest adjustments for future iterations of the protocol in the hopes that my work has laid a solid foundation for a non-fear based observational learning paradigm.

As the mPFC has been shown to support social cognition in addition to a host of other behaviorally relevant functions, my third study used an unbiased approach to analyze neural data in freely moving rats across different behavioral contexts. As most studies of prefrontal areas have been done in well-defined and tightly controlled experimental paradigms on isolated cognitive features, I wanted to break with tradition and characterize neural dynamics in the mPFC in relation to naturalistic behavior itself, that could explain and tie the different findings together. To do so, I wanted animals to be allowed to move freely in different contexts that would prompt them to exhibit a range of naturalistic and spontaneous behaviors, like animals do outside a strictly task-based context. I was able to show that the mPFC carries neural representations at the population level for different behavioral states that evolve dynamically as the animal engages with its environment, potentially recruiting single neurons into meaningful ensembles depending on the momentary needs and motivations of the animal. Physical features and kinematics did not account for the entirety of the behavioral representations, indicating that the internal state of the animal, cognitive processes and/or sensory input from the environment might also contribute. Thus, I show that the mPFC encodes flexible behaviors necessary for solving ecologically relevant tasks, and I use this study as a backdrop to discuss the importance of both well-controlled and naturalistic experiments for a comprehensive understanding of the function of the brain.

List of Papers

Paper 1

Hovde, K., Rautio, I. V., Hegstad, A. M., Witter, M. P., & Whitlock, J. R. (2023). **Visuomotor interactions in the mouse forebrain mediated by extrastriate cortico-cortical pathways.** *Frontiers in Neuroanatomy, 17, 1188808.*

Paper 2

Rautio, I. V., Holmberg, E. H., Kurup, D., Dunn, B. A., & Whitlock, J. R. (2023). **A novel paradigm for observational learning in rats.** *Cognitive Neural Dynamics, 1-11.*

Paper 3

Rautio, I. V., Nevjen, F., Hem, I., Dunn, B. A. & Whitlock, J. R. (2023). **Behavior-state representation in the rat medial prefrontal cortex.** *Unpublished manuscript*

Acknowledgements

The work in this thesis was carried out at the Kavli Institute for Systems Neuroscience at the Norwegian University of Science and Technology (NTNU), under the supervision of Associate Professor Jonathan R. Whitlock and Associate Professor Benjamin A. Dunn.

I would like to thank Jonathan for seeing something in me before I saw it there myself. For giving me the chance to follow my curiosity and develop my passion for learning and development. Your faith in me, the guidance and the freedom you have given me over these years have given me the opportunity to grow as a scientist and as a person. Thank you to Ben, who first was my office mate and introduced me to the land of computation before becoming my supervisor and integrating it even further into my work as I learned to love it. Your patience, enthusiasm and encouragement inside and outside of the lab has been of great support. It is important for me to thank Menno who has shown me so much patience and given so much of his time and knowledge. You truly are inspiring as a scientist and as a person. I would also extend my thanks to the other co-authors of these papers: Karoline Hovde, Andrea M. Hegstad, Fredrik Nevjen, Ella H. Holmberg, Devika Kurup and Ingeborg Hem. Thank you to the members of the lab, current and alumni, for discussions, for the sharing of knowledge and for laughing when I grunt my responses while chugging energy drinks. Thank you to all the other people in other labs at the Kavli Institute, for being open to brainstorming, fruitful discussions and sharing knowledge and for sharing the passion of doing science.

The road to this point has been long but exciting, and challenging but developing. The road has uncovered my own limitations and it has given me the skills, the knowledge and the confidence to surpass them. Growing, learning and developing as a scientist is one thing, growing and developing as a human is a completely different matter. This journey has had that special effect of accomplishing both. I will forever be grateful for this experience. Thank you to my friends and family

for your patience, understanding, support and for being the greatest cheerleading squad anyone can hope for. Specific thanks are warranted. Thank you Karoline for welcoming me so warmly into this world of science and for the friendship that developed. Thank you Heidi for all the talks and the walks and for allowing our friendship to deepen this past year. Thank you Christin for all the coffee and the emotional and scientific support. Our friendship has quickly grown to become very important to me. To Patricia. Thank you for your support, unlimited belief and encouragement. Thank you for reminding me of the colors in life and for grounding my kite.

Chapter 1

Introduction

Imagine you are walking down a busy street in a new city, trying to navigate your way to a goal location - let us say one of many generic tourist attractions you don't really care about but you want to check off the list since you're in this city in the first place. To get to this goal, you would probably be successful if you were to interact with someone and ask for directions. To find someone that might be able to help you and who seems to be approachable you would first need to extract and interpret the relevant information in your surroundings. This would include reading body language when choosing who to ask, deciding on an approach strategy based on their body language, follow local social norms and rules of how to interact and you would encode the received information through different sensory modalities. As this person is explaining, you might visually scan your current surroundings while creating a mental map and performing mental travel of some kind based on the instructions you have received. After leaving your helpful guide, you might even search for a slightly elevated location to get a better view over the area and better orient yourself before venturing out in this new direction with a clear goal in mind guiding your actions.

Now, switch to a new scenario: You are walking through the woods alone with your small backpack and binoculars. You move quietly between the trees, treading carefully, eyes sweeping over the rocks and trees and your ears are perked trying to capture the sounds in your surroundings. Maybe your posture is slightly crouched while moving, trying to appear inconspicuous and non-threatening to the wildlife surrounding you. You've heard from a friend that a small family of elks have been spotted in this area, and you've wanted to see them up close for some time. This is your chance, but you have to choose the appropriate strategy and behaviors to first be able to approach the area they have previously been spotted

in, and then choose a different set of appropriate behaviors to not scare them away. Initially, you need to orient yourself to which direction you have to travel through the woods. To do this you recall instructions given by your friend and you climb up on a big rock as you look for landmarks to navigate by. When you have chosen which way to go you do so at a slow pace, keeping any noise you make to a minimum. You need to be constantly vigilant for different animal sounds, either which animals to potentially avoid or to not inadvertently scare away the animals of interest. Occasionally you might stop and crouch down to eat a snack or you freeze your movements while you are listening and plan your next move. When you are nearing the area where you expect to find the elks, you have multiple options open to you: Do you climb a tree and sit there and wait until they appear? Find a point low to the ground with enough coverage that you can confidently hide and not be seen? Camp down and start a fire because you are starting to get cold? Suck it up and keep quiet and wait in silence? All these action plans are available to you in the current moment, but some of these plans carry with them a higher probability of completing your current goal (spotting elusive elks) than others.

Both of these scenarios carry some similarities: Your physical body as it moves through space; finding higher ground to orient yourself; navigation through unknown environments; using all your senses to gather information and make adjustments accordingly if needed; utilizing stored information from your memory; pursuit of a specific goal which dictates your actions; the option of multiple strategies to reach your goal and needing to choose one of them. Then there are specifics to each context: One is highly social, requiring you to use completely different behavioral strategies than in the other context where you are completely solitary. The physical surroundings are also different, which would impact how you move in your environment as well (slow, deliberate and crouched to move silently through a forest vs. quick small movements as you are winding your way through a crowd trying not to bump into someone too hard or avoid crashing into a cyclist coming towards you at high speed).

These features are all available to your sensory system but are not given similar amounts of attention when navigating your environment. Your current task goal and motivation shape which features are ignored and which are more salient, and with the necessary information to guide you to make the best choice of action selection to reach your goal. Thus, in both conditions you are using a lot of different information streams and neural pathways to correctly navigate physically and socially in the given context. Your current context, defined as your external and internal environment in that given moment, dictates your behavioral state space. A behavioral state space, as referred to in this work, can be conceptually defined as the available behavioral actions you can make in that current moment based on the local affordances and goal for the current task (Kulkarni & Paninski, 2007; Niv, 2019). Task spaces are quite specific in their representation of choices or actions that produce the best performance

on the specific task at hand. An example would be the classic Stroop Test often used to test cognitive control and prefrontal function (Miller & Cohen, 2001). In this test the subject has to respond either according to the color of the letters they are reading, or disregard the color of the letters of the word and instead respond to the color the word itself references. Such as choosing between conflicting stimuli like the word "RED" displayed in blue letters. The current rule decides which of these two task features the subject should attend to. Using cognitive control, a subject thus chooses a weaker but task-relevant response - or attends to a task-relevant source of information - among competing stronger cued responses which are task-irrelevant. A neural representation during this task would be confined to the current rules and your possible responses in this current setting, known as a task space. In this case, the "Stroop test defined task space". On a larger, real-life scale, you have many tasks you need to solve in the context of a larger dynamic environment, hence I use the term "behavioral state space" as I consider this term more appropriate to cover the many potential "task spaces". To define and navigate this state space, sensory, motor and cognitive processes have to be integrated in a brain area and an appropriate plan of action chosen and executed. One of the candidate structures for this integrative process is the medial prefrontal cortex (Sharpe et al., 2019).

The borders of the state space are based on contextual factors, such as whether you are alone in the woods or on a busy street. In the case of the former, interacting with another human being would be outside the borders of your current state space as that choice of action is not currently available to you. It is an irrelevant (non-existing) feature of your current task, and thus does not inform your choices of appropriate actions. Another example from the busy street: you would perhaps focus on the speed on the oncoming cyclist instead of the colorful candy wrapper at your feet, assuming your goal was to navigate while alive (Niv, 2019). What features are most salient in the moment or for performing a task will be expected to determine the neural representation in the relevant brain areas.

To extract these features from the environment, the very first step is to perceive sensory information for the surroundings. Two overarching visual pathways have been hypothesized to inform different major aspects of visually-guided, goal directed behavior: the dorsal “where” and ventral “how” pathways (Ungerleider & Mishkin, 1982), with the latter providing “vision for action” (Milner & Goodale, 1992). The anatomical details for these pathways have been worked out in detail in primates and carnivores, and work over the last 15 years has substantiated that similar pathways exist in rodents (Wang & Burkhalter, 2007; Wang et al., 2012).

One such pathway between higher visual areas and secondary motor cortex (M2) in rodents is reminiscent of dorsal stream pathways in humans thought to transmit information about how to interact with objects (Marshel et al., 2011). Knowing that both motor and extrastriate visual areas also project to prefrontal areas in the rodent brain (Vogt & Miller, 1983; Wang, Gao & Burkhalter, 2010), along with multiple other sensory areas, one logical assumption is that the prefrontal cortex is able to employ this visuomotor information to inform relevant cognitive processes (Miller, 2000; Ridderinkhof et al., 2004; Gilbert & Burgess, 2008). Logically, some of these processes would also underlie more complex forms of behavior, including those related to social cognition and observational learning. It has been shown for rodents that different kinds of observational learning rely on different pathways, for instance, the amygdala-cingulate connections which support observational fear learning (Jeon et al., 2010; Kim et al., 2012; Smith, Asada & Malenka, 2021). In rodents, however, few if any observational learning paradigms depend on learning complex sensorimotor behavioral sequences and, due to this, the pathways which support this kind of non-aversive, non-fear based learning have not been elucidated to the same degree as those relying on fear or physical aversion (Bruchey, Jones & Monfils, 2010; Jeon et al., 2010; Allsop et al., 2018).

The hypothesis that the prefrontal areas are implicated in non-fear-based observational learning comes from the literature showing that prefrontal areas are broadly involved in different types of social cognition across species (Charpentier & O'Doherty, 2018; Carrillo et al., 2019; Olsson, Knapska & Lindström, 2020) and that these areas are highly connected with sensory, motor and "higher" cortical areas (Hanganu-Opatz et al., 2023). The coding flexibility needed to extract the information from the current environment, process and integrate it into a larger whole to generate a contextually appropriate action plan hints at multiple populations interacting and processing across modalities in the same brain area. A coherent theory of the function and coding characteristics of the medial prefrontal cortex during naturalistic behaviors is currently still lacking.

In this thesis, I will present studies that adds to the anatomical literature on visuo-motor pathway in rodents, which are crucial for the brain's ability to extract and utilize external information from the world (simplified schematic shown in Figure 1 with areas studied in this thesis). I present this study first since it posits a pathway by which visual information might enable observational learning – a process which starts with visuomotor transformations of the observed actions of a conspecific. The second study establishes that rodents are able to utilize visual information to learn a novel behavioral sequence vicariously. The last study establishes neurophysiologically how complex natural behaviors are represented in the medial prefrontal cortex.

1.1 Dorsal stream of visual information processing

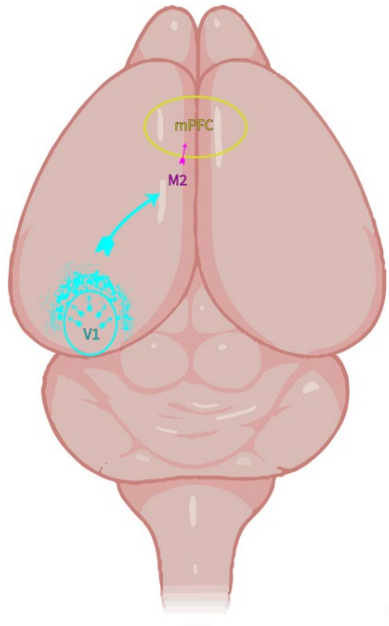

Figure 1. Schematic visualization of the dorsal visual stream in the rodents, by which visual information reaches frontal motor areas. Cyan indicates extrastriate areas (spots), and the pathway from extrastriate areas towards M2 in the mouse cortex. Magenta arrow indicates further anterior projections from M2 to prefrontal areas. Created with BioRender.com.

Having the ability to recognize objects in your environment and knowing how to physically interact with them has traditionally been assigned to two visual pathways in the mammalian brain; the ventral and dorsal stream, respectively (Schneider, 1969; Goodale & Milner, 1992). These two streams have also been known as the "where" and the "how" streams of visual processing (Mishkin & Ungerleider, 1982).

This becomes apparent when we look at which disorders emerge in humans with damage to these associative areas. Patients are consciously able to report descriptors of different objects, like their size, shape and orientation, but are unable to perform the necessary motor adjustments to interact with them in space (Goodale & Milner, 1992). Even if these dysfunctions can be attributed to the damaged area itself, it is probable that the observed effects are also a result of downstream areas receiving

distorted or incomplete information. Thus, as parietal, motor and limbic cortices are downstream recipients of output from V1 (primary visual cortex) and higher visual areas, together comprising a dorsal stream of visual information flow (Wang, Sporns & Burkhalter, 2012), the integrity of the entire pathway is important for normal functioning.

Visual information enters the eye and reaches V1 via the thalamus, and from there the visual signal is transmitted further up the processing chain to higher extrastriate visual areas (Simmons, Lemmon & Pearlman, 1982). There are an estimated 10 extrastriate areas surrounding V1 in rodents, and each of these areas process different spatial and temporal information (Marshel et al., 2011), supporting the hypothesis of functionally different processing streams of visual information. The dorsal stream projects from the extrastriate areas to the posterior parietal cortex (Wang, Gao & Burkhalter, 2011; Wang, Sporns & Burkhalter, 2012), with these particular areas preferentially processing information related to motion and action (Marshel et al., 2011). Certain extrastriate areas, including areas AM, A and AL, were previously shown to project to M2 (Wang, Gao & Burkhalter, 2010), an area important for linking motor actions with sensory cues, similar to the PPC (Young, Stepniewska & Kaas, 2012). Based on these connections, these visuomotor pathways likely support motor coordination and, by extension, movement sequencing while interacting with one's environment.

However, the ability to interact skillfully with objects is quite different, and simpler, than interacting socially with someone else, whether between humans, animals, conspecifics or other agents. Social interaction requires more than just physical or spatial information to be processed. More subtle aspects of one's behavior need to be adjusted to during social interactions, including understanding or predicting the intentions of the other individual, in addition to adjustments to momentary changes in the environment. To guide our actions in space we rely on sensory information like visual input, but action selection toward a goal requires more than just sensory input. In the end, such actions rely on internal models of the world - "representations" - which integrates relevant sensory information, internal state and current contextual rules. Thus, a more elaborate connectivity pattern is necessary. Extrastriate areas in mice have been found to project to M2, Cg1 (also known as dorsal anterior cingulate cortex (dACC)) and IL (infralimbic cortex) in prefrontal cortex and to parietal areas (Wang, Gao & Burkhalter, 2010). Based on this connectivity, Burkhalter et al. (2012) have argued that this is the rodent homolog of the "vision for action" pathway described by Goodale (2011). M2 also projects to cingulate and IL (Vogt & Miller, 1983). In general PL does not seem to have direct connections to M2, but it is connected indirectly as cingulate and IL project to PL, and visual (including exstrastriate), auditory and motor areas project to cingulate (Vogt & Miller, 1983; Reep et al., 1987; Zingg et al., 2014). Together, these may constitute the

main anatomical pathways by which sensory and motor modalities inform cognitive processes in the medial prefrontal cortices, generating the above mentioned representations.

1.2 Mechanisms of learning and different neural substrates

Why is the ability to learn through observation important? First-person learning requires direct exposure to different situations and unknowns which may carry risk for one's own health and safety. In such a situation, the negative consequences of one's actions may result in harm, and in the worst-case scenario even death. If this were to happen, the individual in question would not be able to learn from the consequences of their actions, and their genetic lineage would quickly snuff out if all individuals of that line behaved in the same manner. Thus, observational learning allows the individual to learn the consequences (positive and negative) of certain actions without risking harm to oneself. This ability has been demonstrated to varying degrees in all species tested (Galef, 1976; Galef & Laland, 2005; Zentall, 2012; Loukola et al., 2017) with different forms of behavioral adaptation interpreted as resulting from learning after observation.

Rodents, for example, are able to acquire food preferences by observing conspecifics, either what to consume or what to avoid (Figueroa et al., 2020), and can learn novel strategies to attain food (Del Russo, 1971; Zohar & Terkel, 1991). In addition they can learn navigational strategies (Leggio et al., 2003; Yamada & Sakurai, 2018) as well as fearful and non-fearful associations by proxy (Bruchey et al., 2010; Jeon et al., 2010; Twining et al., 2017). This type of social learning, by which an animal acquires new information, behaviors or skills by observing and interacting with others, happens through different associative processes. Observational fear learning, for example, is dependent upon the observer making the association between a cue (conditioned stimulus example: auditory cue) and the aversive reactions from a conspecific receiving a foot shock (unconditioned stim- ulus: foot shock). This is classical Pavlovian conditioning. This CS-US relationship is strongly and quickly established, presumably because such a negative experience carries with it a strong survival incentive to learn. Therefore, these paradigms are well-established as they produce robust results in a short amount of time, which lends itself to mechanistic behavioral research. Examples of aversive observational learning paradigms are shown in Figure 2. However, this fear-based learning relies mainly on structures in the limbic system (Jeon et al., 2010; Kim et al., 2012; Allsop et al., 2018), which likely do not support the perceptual-motor translations on which observational skill acquisition relies. Skill acquisition would also utilize a slightly different learning mechanism – instrumental conditioning – where the

established association between stimulus and reward prompts the observer to perform a specific behavior to obtain a reward. It has been shown that social transmission of fear and pain states rely on different pathways (Smith, Asada & Malenka, 2021). Thus, it would be reasonable to assume that other pathways outside the limbic system might be necessary for different types of non-fear based observational learning. These other pathways need to be discovered and further investigated to understand different types of learning and their respective neural representations.

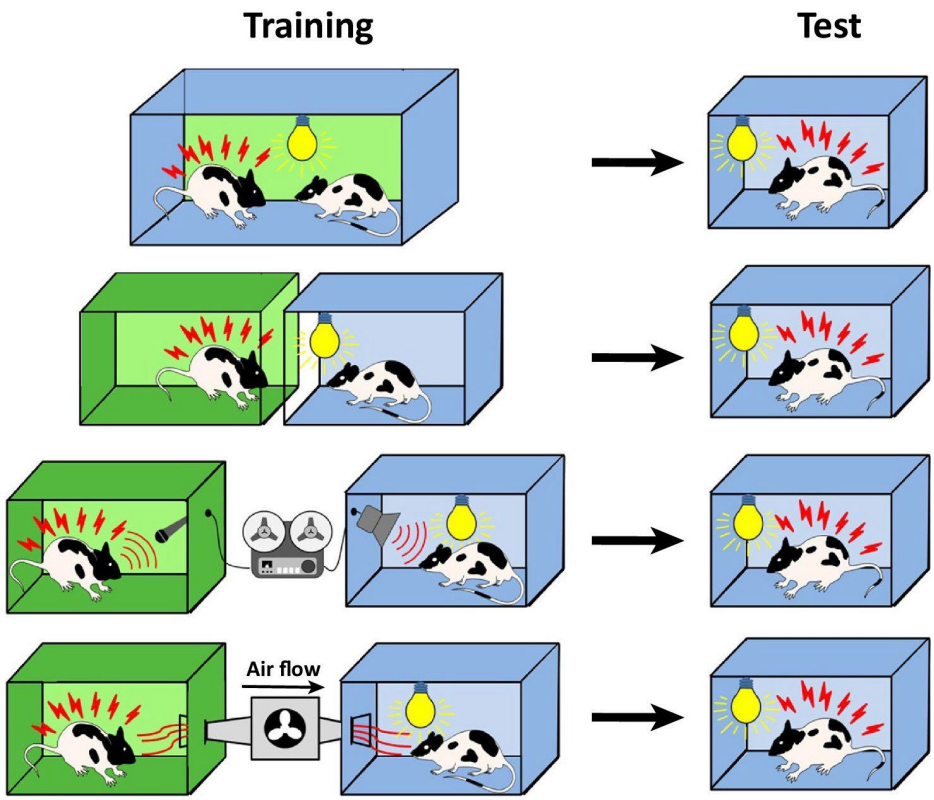

Figure 2. Graphics from Debiec, J., & Olsson, A. (2017). "Social fear learning: from animal models to human function." Each cartoon shows different behavioral approaches for fear-based observational learning.

Learning by observation depends on the brain's ability to transform visual information into appropriate motor signals without the organism first having actually done those specific behaviors. This requires, first and foremost, pathways that enable visual and motor signals to be integrated into a neural representation of the observed action. Then, that representation must be preserved and consolidated to enable retrieval at a later time. During recall, the learned representation is acted out by the observer and scored by an experimenter as evidence that the animal learned the behavior. Where this neural representation is stored, as well as the dynamics of acquisition and recall, are currently unknown.

In the first paper of this thesis, we used high resolution analyses to characterize the physical connections between visual and motor projection neurons in the visuo-motor pathway in the mouse. These connections plausibly enable the integration of visual information with motor signals. The target areas downstream from motor

areas, such as frontal and prefrontal cortical areas which support social and other cognitive processes (Miller, 2000; Mulder et al., 2003; Ridderinkhof et al., 2004; Gilbert & Burgess, 2008; Zhang et al., 2014; Debiec & Olsson, 2017; Charpentier & O'Doherty, 2018; Isoda, 2021; Klein-Flügge, Bongioanni & Rushworth, 2022), would be candidate areas for further investigation.

The second paper therefore aims to establish a novel, visually-based observational learning paradigm that does not depend on fear or deprivation of any kind, opening the door for investigations of novel pathways that support observational learning of behavioral sequences. This paper serves as a proof-of-concept for the paradigm, by which rats learn through observation without the need for motivation by food deprivation or fear of pain. Adjustments are suggested to make future iterations of the paradigm even more robust, which we argue will be important for pinpointing the neural substrates that enable the observational learning of novel behaviors. Such a paradigm would also allow for the investigation of different phases of learning, such as encoding, consolidation and retrieval, and potentially how the relevant types of information are represented in the brain. Additionally, it has been shown that candidate areas like the medial prefrontal cortex contain neurons that encode behavioral information about others and oneself (Isoda, 2021).

1.3 Behavioral representations in the medial prefrontal cortex

Being able to recognize, plan and execute behaviors physically is just one component of navigating one's surroundings. Being able to select and execute the correct physical motions to attain a goal necessitates that a host of mental processes are resolved first. Recognizing which context you are in and which affordances are available, for example, is an initial step useful for establishing the repertoire of behaviors at one's disposal. Then there would have to be weighting between available actions depending on current contextual factors, previous history and memory of actions performed in the context, recalling the outcome of those actions, having a value-system in place to evaluate the pros and cons of potential actions, etc. The ability to recognize and monitor one's current context has been attributed to pre- frontal cortices (Balaguer et al., 2016), as well as the ability to select appropriate actions depending on the environment that the animal is currently in.

"Environment" here includes the physical environment, the internal state and motivation of the animal, as well as the social environment, including one's position in the social hierarchy, etc. All of these features describe an "environment", which can also be defined as a "state space", that serves to constrain the spectrum of potential actions the animal could take for the current task (conceptual illustration in

Figure 3). A state within this space includes all the relevant information needed to make optimal transitions between states to meet current demands (Sharpe et al., 2019). To be able to flexibly change one's behavior according to the current behavioral context, relevant information that distinguishes between contexts has to be extracted first, something for which the mPFC is important (Balaguer et al., 2016; Hyman et al., 2012). A vast network of projections to the mPFC from diverse brain areas (Le Merre et al., 2021; Gao et al., 2022) also likely support computations for immediate adjustments as contextual factors change. It has also been postulated that the neurons in the medial prefrontal cortex exhibit mixed selectivity because this facilitates quick adaptations to evolving contextual demands (Rigotti et al., 2013; Mante et al., 2013; Fusi et al., 2016; Bernardi et al., 2020; Dubreuil et al., 2022; Aoi et al., 2020; Koay et al., 2022).

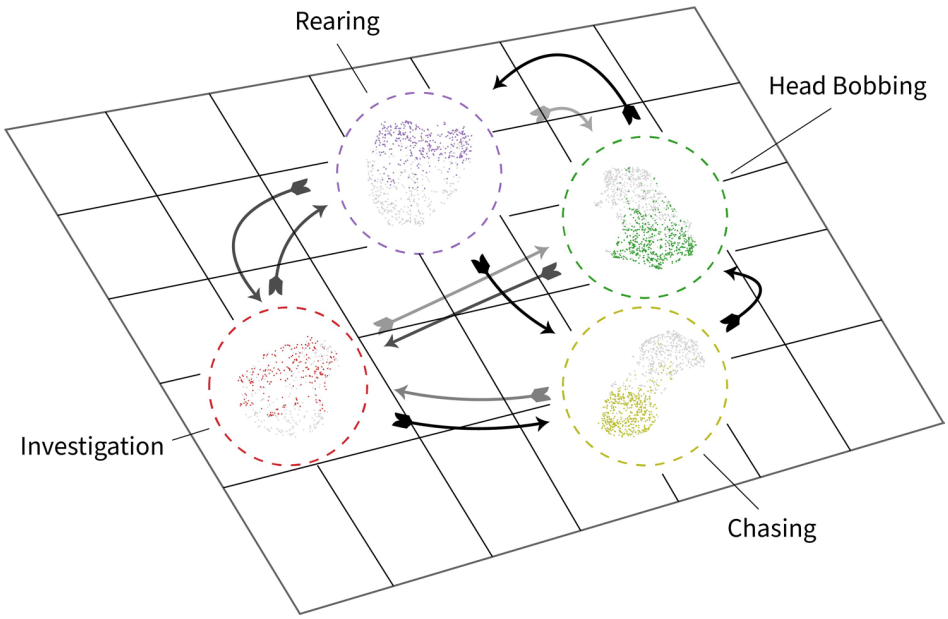

Figure 3. Example of available behaviors (bubbles) in the context of task where the goal is to catch a moving bait (plane), with different options for transitions between behaviors (arrows denote possible transitions; shading indicates transition probabilities).

"State changes" refers to switching between action strategies that are pertinent for the contingencies of the environment at that moment (Sharpe et al., 2019). As certain behaviors reflect particular states or state transitions (Hyman et al., 2005;

Fujisawa et al., 2008; Lapish et al., 2008; Karlsson et al., 2012; Voloh et al., 2023), the neural representations of those behaviors could include the combination of behavioral variables (e.g. kinematics) and internal variables associated with the new state. In line with this view, it has been shown that context-specific movement patterns modulate mPFC activity (Hyman et al., 2012) and non-task related behaviors (such as the trajectory of the animal and climbing) have also been documented in the medial prefrontal cortex (Euston & McNaughton, 2006; Zhang et al., 2022), which suggests that some physical behavioral features are represented in frontal areas of the rodent brain. These studies did not, however, track the animal in detail or check the stability of neural coding of these behaviors outside of a single task-space, leaving the question unresolved as to whether the observed correlates actually were representations of physical movement per se, or of included sensory or cognitive processing related to the task. Aspects of this have been investigated previously in the medial prefrontal cortex, but in within the confines of strictly controlled tasks designed to isolate specific cognitive functions such as attention, behavioral inhibition, memory processes and decision-making (Dalley et al., 2004; Euston et al., 2012; Hirokawa et al., 2019). A recent study that utilized 3D tracking of freely moving rhesus monkeys found encoding of specific sets of actions, such walking and jumping up on an object, in the medial prefrontal cortex (Voloh et al., 2023) and the same areas were also able to predict transitions between these sets of actions. Whether specific behaviors are encoded in the rodent medial prefrontal cortex, and at what level of complexity (i.e. low-level kinematics, discrete actions, entire sequences, etc.), is currently not known in rodents.

The third paper thus aims to explain some of these issues using an unbiased, bottom-up approach to the analysis of prefrontal population recordings gathered from freely-moving animals, tracked in detailed 3D, in different behavioral contexts. By allowing the animals to behave freely in three different tasks, we could investigate prefrontal neural representations of natural behavior and measure whether they depended on the behavioral context in which the animal was placed. By focusing on untrained, natural behaviors, this work brings an ethological approach to the prefrontal coding of behavior, something that to this day has not been thoroughly investigated, particularly in rodents.

Chapter 2

Objectives

To execute any behavior, the first step is to process sensory information from the outside world, which informs which behaviors are physically available to the organism. For this to be possible, the brain requires physical connections between sensory and motor areas for the transfer of information to occur. Sensory information is necessary for all kinds of behaviors, as it is the primary source of outside information that guides action selection in any context. It is, however, an open question how this information is utilized in different contexts and how it ends up being represented in different areas of the brain. One context in which visuomotor information is highly pertinent is during the process of observational learning, a setting where the transformation from visual information to motor output is utilized in a learning process without an immediate motor response. Instead, there is a process of consolidation, storage and recollection of that information for later use, resulting in appropriately timed motor output if the learning was successful. Areas that have been shown to be important for observational learning include the prefrontal areas. The neural representation of different behaviors in these areas has only begun to be explored in freely-behaving subjects. In highly controlled cognitive tasks, the prefrontal areas typically show unstable tuning to task features at the single cell level, with stability emerging at the population level. Whether this also holds for naturalistic behavioral patterns in unrestrained animals, and rodents in particular, has not been investigated before. Some research about how naturalistic behavior is represented in the medial prefrontal cortex has recently been published, but as of yet no one has investigated this to the level of detail as has been done in the work presented here. Most studies show behaviorally dependent state changes in either single trials or single sessions, but within the same task setting and with minimal control for physical features that might explain these changes in neural

activity. Such examples could include movement speed, turning of the head or bodily and postural components.

In this thesis I aim to elucidate this broad area of research with individual papers, each having their own objectives to answer different questions of how contextual information enables and reflects the neural representation of different behaviors.

1) Paper 1 aims to identify where in mouse visual and parietal cortices there is colocalization of V1 output and M2 input to refine existing knowledge about the visuomotor pathway in mice. By reconstructing single cells in 3D, I aimed to identify more precisely not just where colocalization of M2 projecting neurons and V1 fibers occurs, but to ascertain whether there are in fact putative synaptic contacts between them as well. This is done by estimating sufficient proximity between pre and post synapses for synaptic transmission using high resolution 3D reconstructions made using stacked confocal images.

2) Paper 2 aims to establish a non-aversive learning paradigm that relies on the visuomotor pathway, such that rats learn a specific motor sequence purely through visual observation of a conspecific. It is not sufficient for visual information to only be perceived, processed and encoded correctly, but it has to be stored and updated over three consecutive days before retrieval of the learned motor sequence on testing day. This paper shows that this process of transformation, storage and retrieval of complex visuomotor information has taken place, as reported through adaptive behavior. The novelty of this paradigm is that the animals learn through observation without being motivated by fear or food deprivation, and the task the animals learn is a 2-step behavioral sequence, as opposed to classical conditioning to a cue that is associated with a simple and immediate response.

3) Paper 3 aims to elucidate the neural representation of complex rat behavior in the mPFC, one of many areas with connections to the visuomotor pathway and associated areas. This paper establishes that even in a freely-behaving animal, stable behavioral representations are found in the mPFC, and these behaviors have neural signatures that separates them from each other. There is coding for both context-specific behaviors as well as behaviors that generalize across physical environments. The experimental design allows for testing the robustness of the neural responses as the animal moves through different behavioral states in different contexts, shedding light on whether behavioral representations are purely kinematic or if they reflect internal processes specific to the mPFC.

Chapter 3

Synopsis of methods

3.1 Paper 1

Nine C57BL/6JBomTac mice (eight female, one male) were used in the first paper to show colocalization between V1 fibers and M2-projecting neurons in extrastriate areas. Adenovirus and anterograde tracer were injected into secondary motor cortex (M2) and primary visual cortex (V1), respectively, in the left hemisphere of five animals, with their brains later being cut in tangential flattened sections. Out of these five, one brain was excluded from final analyses due to poor tracer uptake. The tracer injected into V1 was an anterograde tracer, 10 KD biotinylated dextran amine (BDA) and the virus injected into M2 was a retrograde GFP-tagged adeno-associated virus rAAV2/1-retro (retrograde AAV-CAG-GFP; serotype "retro"). The four remaining animals received similar injections in M2 and V1 but in the right hemisphere, and their brains were cut in coronal sections. Due to a misplaced injection in V1 one brain was excluded from the final analysis.

21 days post-injection all animals were euthanized and transcardially perfused using freshly prepared ringer's solution and PFA (1%), after which the brains were stored in a cup of PFA. The left hemisphere of the cortex was dissected out within 1 hour and flattened, before preparing (50 μm) tangential sections. The hippocampus was unfolded, and the cortex was flattened between two microscope glasses and submerged in PFA (4%) overnight at 4°C, followed by a cryoprotective solution the night after. The flattened cortex was then cut in 50 μm tangential sections in one series on a freezing microtome. This series was processed to reveal the BDA tracer and to enhance signal from the virus using a 2-day immunohistochemical procedure. The same perfusion procedure was performed on the animals with injections in the right hemisphere, and were subsequently stored in a PFA-filled (4%) container overnight and a

cryoprotective solution the night after. The brain was cut on a freezing microtome in 40 μm sections in three series. The first series was processed with Nissl-stained, the second was processed to visualize the tracer and virus in the same way as for the series from the tangential sections, and the third series was stained with DAB against M2AChR2 to visualize M2AChR density and were used only for delineation purposes. If the third series was not used it was kept as a backup in a cryoprotective solution and stored at -24°C. All Nissl and M2AChR stained sections were digitized with a bright field scanner for further analyses. Sections with fluorescence labeling were examined in a fluorescence microscope and digitized with a fluorescence scanner, from which selected sections were scanned with a confocal microscope to acquire high resolution images (63× oil) in z-stacks (typically 70-90 planes, 0.14 μm intervals, 0.05 μm pixel size). These images were then convolved before being manually reconstructed by tracing in 3D, after which the synapses on reconstructed dendrites and axons were tested for close proximity, and were defined as forming putative synapses if they were within 0.25 μm of each other.

3.2 Paper 2

16 male Long Evans rats were used in the second paper of the thesis. All animals were implanted with a stimulating electrode targeting the medial forebrain bundle (MFB) at the level of the hypothalamus in the right hemisphere. Intracranial self-stimulation was used as the reward in this instrumental learning paradigm. The animals were randomly assigned roles as performers or observers. Performers were trained on a 2-step behavioral sequence where they had to tap a lit sphere - without getting rewarded - to trigger the lighting of a second sphere on the other side of the experimental chamber, which they then were rewarded for tapping. Tapping of unlit spheres yielded no reward and no punishment. When the performer animals performed this task to criterion, a naive animal was placed in an adjacent chamber - separated by a perforated barrier - to observe the performer executing the learned task for 30 minutes a day over three consecutive days. The observer animal was also implanted with a stimulating electrode targeting the MFB, such that whenever the performer animal successfully performed the behavioral sequence both animals received positive stimulation as a reward. On the fourth day the observer animal was put in the experimental chamber and tested for learning. If the animal had learned the behavioral sequence through observation, they would shortly after the initiation of the session start tapping the spheres in the correct sequence. Animals that did not show learning either did not interact with the spheres to a meaningful degree or tried interacting with the spheres but failed to perform

the correct behavioral sequence when the spheres were lit. After the experiment was done, observer animals were further trained to be re-used as performers for subsequent experiments. At the conclusion of the experimental series or when animals stopped performing to criterion, the animals were perfused and the location of the stimulating electrodes were confirmed.

3.3 Paper 3

3 female Long Evans rats were used in the third paper of this thesis. The animals were implanted with Neuropixels 1.0 probes in the right hemisphere, targeting the medial prefrontal cortex (mPFC) and it's three subregions - infralimbic (IL), pre- limbic (PL) and cingulate (Cg) cortex. After implantation the animals were cycled through a repeating sequence of three behavioral contexts: An elevated linear track, an open field foraging task, and a chasing task. All animals went through the three behavioral contexts in the same sequence three times, for a total of 9 recording sessions for 10 minutes each per animal. The animals were not trained in any of the contexts and were free to move and behave as they wished. The elevated linear track had the addition of a second animal placed on the other end of the track, but separated from the animal being recorded so as to avoid damage to the implant. The open field had crumbs of food spread out to encourage movement and foraging, but the animal was free to chose other behaviors available to it in the empty arena. In the chasing task the experimenter prompted the animal to chase after bait on the end of a fishing line. Once the animal caught the bait and consumed the reward, a new trial of pursuit was initiated. Animals chased the bait naturally, without previous training or need for food-deprivation, and were thus free to engage in or disengage from the behavior. Each of the three behavioral contexts were designed to have unique and overlapping behavioral options available to the animal. During these behaviors electrophysiological recordings were made from the mPFC and the animals were all tracked in 3D with retroreflective markers placed on their head and back. The secondary animals on the elevated track were marked and tracked the same way. The trunk was tracked with markers placed at three points - tailbase, back and neck - and the head was tracked with a four-point rigid body affixed to an in-house designed cap that also provided a protective casing around the implanted probe. This experimental design allowed us to record neural activity and keep track of each single neuron, and then map the neural activity onto detailed pose and movement features across all three behavioral contexts. At the end of each experiment the animals were promptly perfused, and location of the probe was confirmed histologically.

Chapter 4

Synopsis of results

4.1 Paper 1

Substantial evidence of a visuomotor pathway comprising a dorsal stream of visual processing in mice have been put forth in prior work (Wang et al., 2011, 2012; Zingg et al., 2014), but many of the details of the pathway remain unknown. Connectivity is usually inferred when afferent fibers and dendrites are colocalized within an area, but this is not sufficient to claim synaptic connectivity actually exists. The distance between synapses is crucial, as fibers and dendrites may colocalize in an area but if they are too far apart, no communication is possible. In this first paper, we further characterized the visuomotor pathway in mice with a dual anterograde and retrograde labeling strategy, investigating the connectivity and intersection of V1 output fibers onto M2-projecting neurons in mouse extrastriate cortex. In flattened brain sections the dual labeling strategy indeed confirmed that visual and motor pathways overlapped in extrastriate areas and posterior parietal regions, which supports visuospatial and motor behavior in rodents, consistent with a dorsal pathway for visuomotor processing as identified in rodents (Wang et al., 2011, 2012).

This experimental protocol allowed us to generate high-magnification reconstructions of the M2-projecting neurons and the V1-fibers in high resolution, permitting distance analyses between synapses. Colocalization analyses were performed between V1-fibers and M2-projecting neurons, and putative synaptic contacts (*i.e.* connections within 0.25μm) were identified in anteromedial (AM), posteromedial (PM), rostrolateral (RL) and anterolateral (AL) extrastriate areas in flattened brain sections. The 3D reconstructions on the whole revealed that most putative connectivity occurred in both superficial and deep layers of the extrastriate areas but

was markedly higher in layer 2/3. The deeper layers had much sparser distribution of V1 fibers than the superficial layers, as well as a lower amount of putative synaptic contacts as revealed when further quantified. In the coronal sections and at lower magnification, the labeling did indeed span both superficial and deep layers, but became progressively more superficial at more posterior brain sections.

4.2 Paper 2

Observational learning has been demonstrated behaviorally across all species tested, and is of evolutionary high value as it allows for learning without risking harm to the individual, and thus their genetic lineage, improving evolutionary fitness. Laboratory studies have amassed data on observational learning mainly using fear-based paradigms or by using deprivation as motivation for task-learning. These paradigms are robust precisely because they tap into mechanisms and pathways important for survival of the animal, but they are limited in that fear-based associative learning is largely limited to hippocampal and limbic circuits for further investigation. Non-fear-based types of learning have been underrepresented, particularly in rodents, due to the challenges of creating robust experimental paradigms that would draw upon more cognitive- or perceptually-based learning processes. In the second paper I present a novel experimental paradigm that does not depend upon fear or any kind of deprivation to motivate the animal to learn a 2-step behavioral sequence through observation. The paradigm is purely reward-based, in that the animals learn to tap lit spheres in a specific sequence by watching well-trained demonstrators, where both observer and performer receive rewarding intracranial stimulation with every successful trial. We found that stimulating the medial forebrain bundle as the reward was key to making this experimental paradigm effective, since it sustained the animals' motivation and task engagement for the duration of the experiment. After three days of observation and a 24-hour delay after the last observational session, the observer animals outperformed control animals on multiple metrics of efficiency and task performance indicative of having learned the task. A subset of animals did not learn the task after three days of observation, which is why we suggested adjustments to make the protocol even more robust in future iterations. Having an even more robust paradigm would allow for moving beyond mere behavioral metrics and into perturbation and electrophysiological experiments. We claim that increasing the observation days to five instead of three would be one of the most important adjustments, based on results from earlier iterations of the task. Preliminary experiments with chemo- and optogenetics also indicated prefrontal areas as potential loci for the acquisition, consolidation or recall of this form of observational learning. This gives fertile ground for diving

deeper into the neural coding of visuomotor transformations underlying vicarious learning, or the representation of behavior itself in prefrontal areas. This also motivated the third project.

4.3 Paper 3

The medial prefrontal cortex has been implicated in a diverse set of cognitive functions in both freely-moving and head-fixed animals (Miller, 2000; Ridderinkhof et al., 2004; Dalley et al., 2004;Gilbert & Burgess, 2008; Wu et al., 2017; Le Merre et al., 2021), with consistent findings of unstable tuning at the single-cell level, but stable population codes (Bernardi et al., 2020). Even with a large knowledge base describing prefrontal functions, a coherent framework that unites and explains the previous findings has long been lacking. The field has long relied on rigorous, tightly controlled experimental paradigms since they bring a level of structure that heightens the "signal-to-noise" ratio of neural tuning reflecting specific functions. However, this also deprives the brain of natural sensory input that would come from bodily movements in dynamic environments in which the animal would naturally behave. This removes input to the brain that would affect processes for action selection and behavioral adjustments (Parker et al., 2020), thus eliminating certain neural dynamics from the data. Even when comparing naive versus trained animals it has been found that the brain dynamics are different after having learned a task in a head-fixed paradigm (Peters et al., 2022; Arlt et al., 2023), making extrapolations to freely moving animals and more general brain function tricky. We were also motivated by the idea that the vast diversity of task designs partly explains the diverse findings in the field, we chose in the third paper to record from freely-behaving, untrained animals in a three-task paradigm in which we tracked the animals' bodies in detailed 3D while acquiring high density recordings from the medial prefrontal cortex.

This approach enabled us to keep track of individual neurons across all three tasks and allowed for comparison of neural tuning to different behavioral features in different behavioral contexts. As the animals rotated between an open field foraging task, a chasing task and an elevated track with another animal available for social interactions, the different environments afforded the animal different options for engaging in behaviors that were task specific (*e.g.* foraging, pursuing a moving bait and interacting with another animal). However, as the three contexts also contained similarities (*e.g.* the open field foraging and chasing tasks were both in the same arena), this enabled comparisons of neural tuning to the same behaviors (*e.g.* rearing, investigation). Using a behavioral classifier we defined and labeled discrete

behaviors which we termed "behavioral states", which enabled investigation into the associated neural representations. This was done by generating co-activity matrices for all possible pairs of neurons during each identified behavior. By plotting the mean co-activation between each pair of neurons during each of the identified behaviors we found unique population states that differentiated the behaviors from one another (“thumbprints” of co-activity). We found that the behavioral state of the animal was modulated by the context of the environment, with the behavioral state of active pursuit being one of the strongest findings across all three animals, but that there were general behavioral states that was stable across contexts as well. We describe this as the immediate environment and the momentary state of the animal creating a behavioral state space to act within. Thus, the neural dynamics do show some change, as the boundaries of the behavioral state space are different in different environments that provide different behavioral options (*e.g.* having a wall to climb or a bait that appears intermittently to chase).

We identified context-specific and -general behavioral states within and across all three environments that could only partly be explained by the physical movements of the animals. As with previous studies (Lapish et al., 2008; Mante et al., 2013), we found unstable tuning at the single cell level and stability at a population level, but the data further suggested that this apparent instability may have reflected that some units were either mislabeled ("speed" cells may not have been truly tuned to speed, but partook in an internal representation of “pursuit” which was associated with high running velocities) or were inconsistently recruited by neural populations during specific behaviors, potentially depending on internal processes or the momentary state of the animal. Our work here shows that the observed neural dynamics in the mPFC appear to reflect the current behavioral state of the animal, which is determined by the context the animal is in - both external and internal. We thus argue that both highly-controlled, task-based experimental paradigms and naturalistic approaches are both important for understanding the brain and that they complement each other, given that one is aware of how the approaches can influence the dynamics the brain. Even in areas that have traditionally been associated with internal and cognitive processes, we find evidence for behavioral modulation, indicating that we cannot assume a dissociation between the physical aspects of the body from the computations of the brain when designing experiments.

Chapter 5

General discussion

5.1 Visuomotor information streams and action selection

The very first step in interacting with the external world begins with receiving information through one or more sensory channels, and vision is one of the primary sensory modalities for gathering information about the environment. In order for this information to guide behavior, it must be broadcast from visual cortices to many areas. Two main cortical output streams of visual information processing are usually mentioned in this context: the dorsal and ventral streams. These streams have traditionally been considered to guide spatial and visually guided motor behavior and enable object recognition, respectively (Goodale & Milner, 1992; Nassi & Callaway, 2009). Even if further subdivisions and nuances have been proposed for these pathways (Sedda & Scarpina, 2012), their conceptual division is helpful in understanding how the brain is able to relate to the external world. Visual projections to cortical areas important for motor control in rodents are reminiscent of the traditional dorsal stream (Itokazu et al., 2018), with some of the same motor regions further projecting to prefrontal areas (Bedwell et al., 2014) that have been implicated in behavioral flexibility and action selection (Dalley et al., 2004; Laubach et al., 2015; Sharpe et al., 2019). This is also reflected functionally as activity of individual V1 neurons have been found to be correlated with activity in ACC, motor and somatosensory cortices (Clancy, Orsolic, & Mrsic-Flogel, 2019), indicating that these areas are able to utilize visual information for further downstream processing. Our work corroborates the putative connectivity between extrastriate areas and frontal motor areas, using high-resolution 3D-analyses which had not previously been brought to bear on this pathway. One of the gold standard methods to show that a synapse is present is to visualize it with electron microscopy, which we did not have at our disposal for this particular study. However,

previous work using electron microscopy and immunostaining collected convincing evidence showing that putative axonal and dendritic connections at distances of 0.25-0.30 μm were highly like to be genuine synapses (Czajkowski et al., 2013). As 0.25 μm was our criteria for labeling markers as likely synapses, we are confident our findings reflect actual colocalization between V1-fibers and M2-dendrites. Detailed 3D-reconstructions made from z-stacked confocal images of individual M2-projecting neurons (see example in Figure 4) were necessary for these analyses, but the analysis was done on only a subset of all identified cells, so the results may have been biased in other ways. For example, the identified distribution of putative synaptic connectivity in the different areas could have been biased by the location of the injection sites in V1. This does not invalidate our results, but we simply cannot claim to have fully characterized the nuances of this particular visual-motor pathway in light of limitations - both in relation to injections and the volumes of tissue analyzed. The anatomical substrates that allows for communication between visual and motor areas are thus confirmed with our study, and this study adds that much of the feedforward visual-to-motor signaling occurs at least via abundant synaptic connections in layer 2/3 of anterior and medial extrastriate cortical areas.

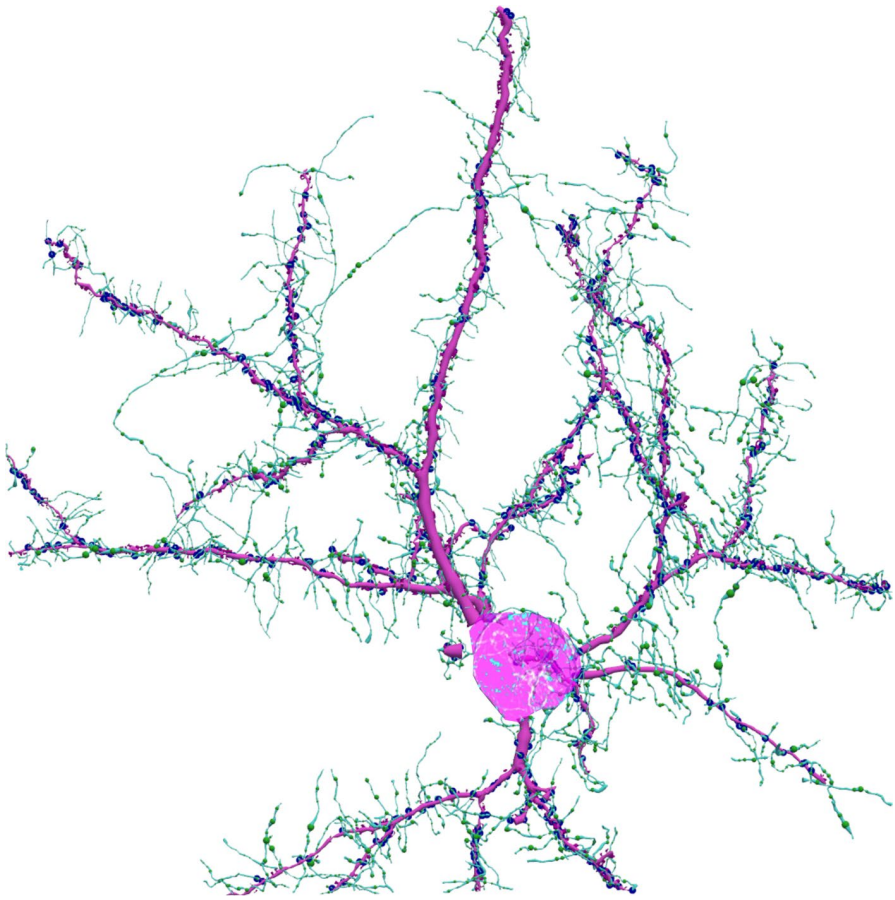

Figure 4. 3D reconstructed M2-projecting neuron from extrastriate area AL in magenta, V1-fibers in cyan, putative synapse as dark blue circles, and putative pre-synaptic varicosities as green spheres.

To interact with one's environment effectively, the physical attributes of one's surroundings are first extracted from visual information, and that information guides which behavioral options an individual can choose from. These affordances, or opportunities to manipulate or use objects in the local environment (Gibson, 1977), are part of defining the current behavioral state space of the animal. The affordances of an object or an interactive agent, the properties that define the use or interaction, are mainly dependent upon physical attributes which must first be perceived or seen to inform possible motor acts. The dorsal stream supports these computations not just for physical aspects of the object or interactive agent (shape, spatial location, size, etc.) but also for

features of the environment that support appropriate action selection that are not restricted to vision (Sedda & Scarpina, 2012). The immediate behavioral state space of the animal, meaning the behavioral options available in the current environment, is further defined by the motivation of the animal itself, its internal state, past experiences with similar contexts, etc. Something like this putative pathway of visual-to-motor-to-cognition would be necessary for navigating and adjusting to changes in one's current context and to use available information to learn from the environment.

5.2 Observational learning, new paradigms and neural substrates

As mentioned in the introduction, observational learning has been studied mostly in the context of acquired fear (Jeon et al., 2010; Bruchey et al., 2010; Twining et al., 2017), which has yielded valuable and robust results elucidating the neural pathways and underlying neural circuits, primarily in rodent models. However, as these paradigms rely on negative emotions or avoidance behavior, the potential neural substrates of relevance are restricted to limbic and related areas. The field has thus long been lacking paradigms allowing for a broader investigation of other forms of observational learning. With our paradigm described in the present work, we not only show that rats can learn a 2-part motor sequence through observation, but that they can do so without being motivated by fear or being deprived in any way. This opens the door for investigating neural substrates of observational learning that likely rely on visuomotor pathways, or investigation of other cognitive features like the different steps of learning through observation. Even if the paradigm in it's current form isn't as robust as it could be made to be, the protocol as published is a proof of concept. With a few adjustments, such as increasing observational days from three to five, we believe this paradigm holds promise to contribute greatly to the field of observational learning (Figure 5 illustrates the physical design of the paradigm).

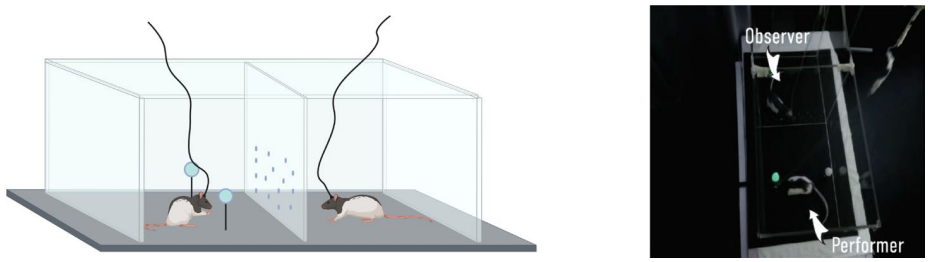

Figure 5. Visualization of task set-up for non-fear based observational learning.

Unpublished results using chemogenetic and optogenetic approaches to inactivate parts of the medial prefrontal cortex of observer animals during each observational sessions indicated that the frontal cortices were heavily involved in either the acquisition or consolidation stage of the learning process. Whether the mechanism that was perturbed was the animals' ability to correctly perceive and understand the task, or if it was an inability to consolidate the knowledge during the intervention is unclear. Further experiments with more temporally specific protocols for disrupting the function of the medial prefrontal cortex would be necessary. What this finding did inspire, but which we were unable to pursue, were electrophysiological recordings in the medial prefrontal cortex over the entire experiment. As the protocol has clearly defined epochs, like the naive stage before any observation, single sessions of observation each day and the final day of testing, there were multiple time points where one could perform electrophysiological recordings and to chart the neural representation of the learning process.

One interesting approach would be to record the animal during a baseline session before any observation has occurred, then record each session for a set amount of observational trials and test the animal on a final day, simply see if the animal had learned the task or not. When analysing the recordings afterwards, one should be able to differentiate between activity in the first observational session that is correlated with the act of observing a task-performing conspecific from baseline activity. Comparing across the observational sessions, one might the be able to see event-related changes emerge in the neural dynamics that indicates when learning has occurred, either during one of the training sessions or the final day. This could be used to test the hypothesis that the same changes in the neural dynamics would not be present in animals that did not learn the task. Sustained changes in firing rates related to rewarded actions have been found to develop in parallel with learning in first person instrumental conditioning paradigms (Mulder et al., 2003), but whether the same changes would be observed when learning occurred through observation is unknown. Assuming this approach was successful, one could go one step further with a final follow-up experiment to test whether the

observed neural dynamics of learning truly reflected these processes. To do so, one could collect the same baseline recording as described above and record across observational sessions, but this time one would not have a predefined number of sessions before testing. Rather, one would do analyses of the recorded data in between each of the observational sessions and only test the animal when the previously identified changes in neural dynamics became apparent in the data. Such a neural change could be firing rate changes that emerge and stabilize during the observation of ongoing trials, like the Mulder et al. (2003) study found. Network wide coordinated firing rate changes have also been previously shown after introducing environmental changes during an ongoing task, interpreted as a change in the animal's internal representation of the success probability of a behavioral option (Karlsson et al., 2012).

Another approach would be to record and investigate the behaviors that the particular environment facilitates in the animal and their neural representations, examining potential changes in these representations or states that the animal is in. State changes could reflect a change in behavioral strategy, which is observed physically as different behaviors with (presumably) different motivations and expectations of different outcomes dependent on the current context and behavioral state. A couple of different behavioral states, possibly reflecting different behavioral strategies, are observable in the observational learning paradigm. Obvious state differences that would have been interesting to record electrophysiologically are the different states of "observing" and "performing". One would assume these two states with their contextually appropriate behavioral patterns would have different neural representations in the medial prefrontal cortex. There are, however, also subtler instances of transitions between states in this paradigm. During the actual performance of the task, the animal is in one state as they go to tap the first sphere, at which point the animal transitions to another behavioral state that is contingent upon the previous one. The new state carries with it the same behavior (approach a sphere and tap) but with different expectations associated with the behaviors (in the first state there is no expectation of reward when tapping the first sphere, since the task requires an unrewarded tap to permit a rewarding second tap, so the second state would carry reward expectation). If these assumptions are true then, based on our findings in the third paper, we would expect to see some overlap between single units in these two "sphere-tap-states" but with information about the expected reward influencing the structure of the population code, reflecting a transformed state space. The same test could be conducted in the brain of an observer, to determine if state space changes occur when the animal learns to expect reward based on the actions of the demonstrator.

5.3 Neural representations of behavior in the medial prefrontal cortex

Usually when investigating medial prefrontal cortex function, the tasks are designed to test a specific cognitive function with a high degree of control over stimulus timing, cues, behavioral responses, task-transitions, etc., especially in tasks which include timed behavioral responses or action initiation and inhibition (Dalley et al., 2004; Laubach et al., 2015; Hirokawa et al., 2019; Sharpe et al., 2019). Recently more focus has been put on naturalistic recording conditions (Smith, 2023; Voloh et al., 2023; Maisson et al., 2023), reflecting an increasing awareness that brain activity is impacted by physical context (Lee et al., 2022), the degree of training (Peters et al., 2022) and the posture of the animal (Mimica et al., 2018; Mimica et al., 2023). In general, tasks using behavioral restraint in which the animals are overtrained to perform actions that are not ecologically relevant, the task or learning itself might elicit neural responses that never occur in natural behaviors, leading to skewed conclusions about the actual function of a system in the brain. Some studies utilizing more naturalistic task designs with freely moving animals have been able to capture different states in the medial prefrontal cortex that can be associated with phases and transitions in the learned task, such as hide and seek (Reinhold et al., 2019; Bagi et al., 2022). However, such studies often lack control or do not monitor carefully the physical behavior of the animal. Behaviors and transitions in many instances are identified by human eyes when examining video recordings. Additionally in relation to this lack of control, the states are assumed to reflect the task phases and internal processes without the ability to disprove alternative explanations. With broadly defined states and imprecise task phases and transitions, it is hard to claim with confidence what the data is actually showing.

In our work we sought to control for as much of this as possible with detailed 3D tracking of the animal, which was helpful in discerning whether single cell tuning or population states were reflective of something in the current environment of the animal or related to features of the head or body. The tracking also allows us to have a clearly defined start and end point for different behavioral epochs to map to their behavioral states. This rigorous tracking and control reduces the noise that comes with human experimenters in our analyses. As we are not able to draw conclusions about the cognitive processes that might occur during the different behavioral states we identified, we are careful with our claims of what exactly the different neural dynamics represent in their entirety. What we show is that the neural activity in the medial prefrontal cortex is partly modulated by some physical features, but this is not sufficient to explain the entire population structure, which suggests contributions from other, untracked sources (Figure 6).

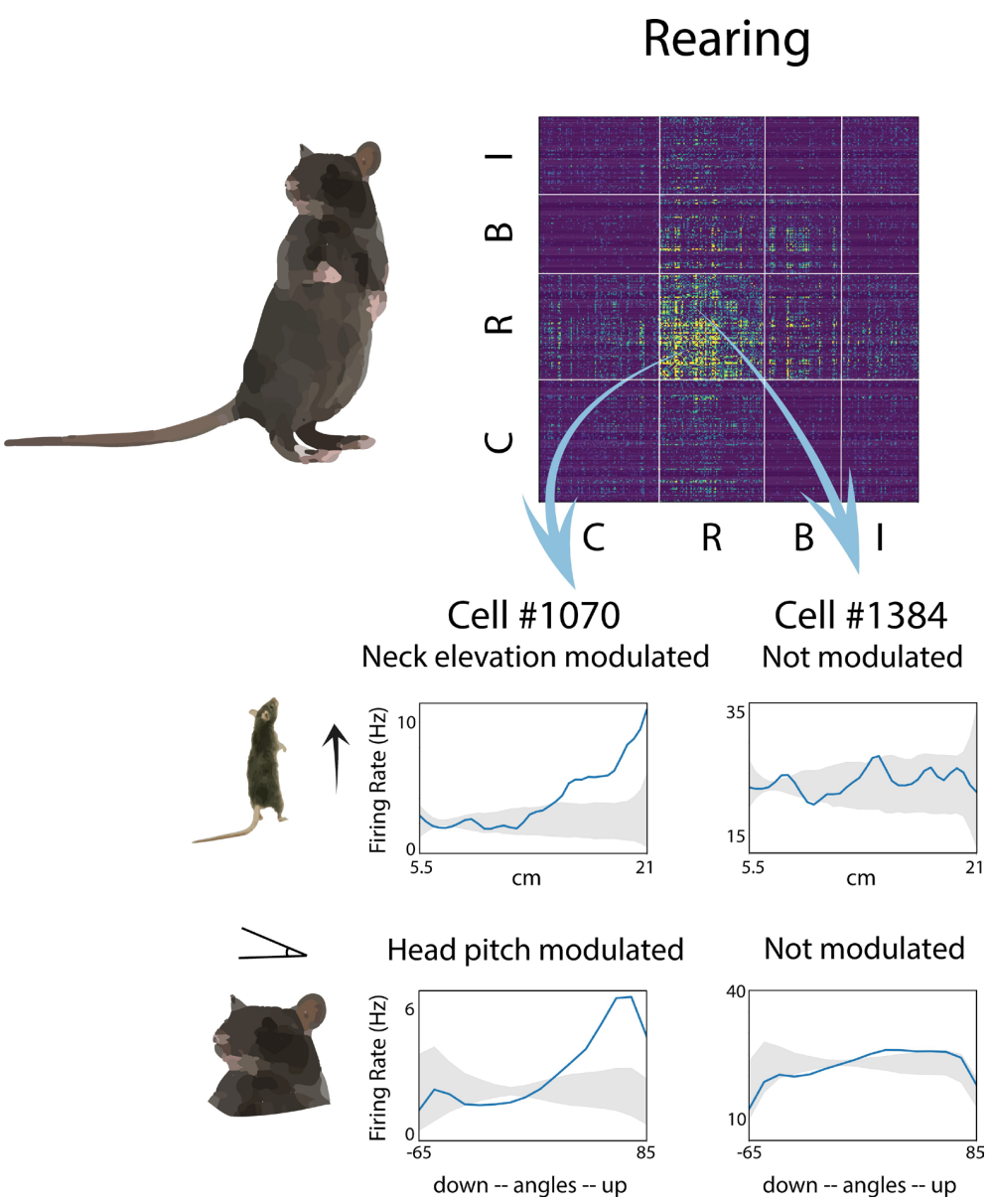

Figure 6. The behavior of rearing is not fully explained by the physical components or the kinematics of the behavior at the single cell level. Some cells were significantly tuned to the act of rearing without exhibiting any sensitivity to physically relevant features of the behavior.

Anatomical studies have established that different subdivisions of the mPFC receive input from visual, auditory, insular, temporal, parietal, somatosensory and motor areas, and that the different subdivisions of anterior cingulate, PL and IL are also heavily interconnected (Reep et al., 1987; Heidbreder & Groenewegen, 2003; Bedwell et al., 2014; Hanganu-Opatz et al., 2023). This connectivity allows for integration across multiple modalities required to acquire, interpret and adjust ones behavior to the current demands of the environment, creating a momentary behavioral state of which we argue we have captured a neural thumbprint (e.g. "rearing", as shown in Figure 6). The recruitment of different units at a given time would thus depend on the current behavioral state space and motivations of the animal. In a moment of, for example, seeking to acquire environmental information by rearing (Lever et al., 2006), the physical movement associated with the behavior, the body posture, the associated cognitive processes and the internal state of the animal would all be represented together in that moment in the medial prefrontal cortex. In line with this, it has been shown that cortical neurons can be recruited in a context-dependent manner and that the behavioral state flexibly modulates cortical function (Cardin, 2019). Thus, different units could be recruited at the same time and together constitute a rearing ensemble, which is what we see in our recordings, as only a fraction of the rearing units are explained by physical attributes. Other kinds of unit that are part of the rearing ensemble are head direction units, which is consistent with rearing being an act of gathering information about the environment, and thus the animal (and network) need to know in which direction they are facing. The larger, unexplained, part of the rearing ensemble would plausibly be explained by untracked internal states or ongoing cognitive processes related to processing external and internal signals.

State- and context-dependent changes in ensemble activity have been demonstrated in multiple motor and sensory cortical areas (Cardin, 2019). As the mPFC is an associative area with highly diverse connections with the rest of the brain (Hanganu- Opatz et al., 2023), other factors that might contribute to the neural dynamics we observed could include sensory input, internal signals (Azzalini et al., 2019; Tallon-Baudry, 2023) or cognitive processes (Miller, 2000), though we did not have access to these in our data. We did, however, see that single units were recruited into behavioral ensembles that reflected the momentary state and apparent interests of the animals, with overlap in tuning, as seen, for example, with cells that were dually modulated by head direction and rearing.

5.4 Head direction signals in the rat medial prefrontal cortex

One of the unanticipated findings we discovered by virtue of our detailed tracking of the animals' heads were single units with strong tuning to allocentric head direction. Head direction cells are single units that show a strong preference for a specific direction in allocentric coordinates, meaning the animal's head direction in relation to an external reference. Since this has not been shown in previous literature in rats (few have looked for head direction cells in medial prefrontal areas, with little success (see Poucet, 1997 and Jung et al., 1998)) and only recently shown in non-human primates (Maisson et al., 2023), we did not try to isolate this variable in our experiments a priori. Even if the cognitive processes associated with prefrontal areas support navigational ability (Hok et al., 2005; Patai & Spiers, 2021, Maisson et al., 2022) this particular feature has not been investigated intensively in rodent prefrontal areas as it has in the more traditional areas like the hippocampal formation (Taube et al., 1990) and thalamus (Jankowski et al., 2014; Peyrache et al., 2019). Cells with spatial tuning properties have been reported in several systems outside of the traditional areas (Wikenheiser et al., 2021; Basu et al., 2021; Long & Zhang, 2021; Poo et al., 2022), so it is not unprecedented to have found cells encoding navigational features in other areas as well, as was the case in recent work in non-human primates (Maisson et al., 2023).

There are also direct anatomical and functional connections between the medial prefrontal areas and classical head direction areas (Guldin et al., 1981; Ferino et al., 1987; Fujisawa & Buzsáki, 2011) that could support this form of tuning in the medial prefrontal cortex. We found cells with the clearest and most definitive head direction cells in one animal, and weaker tuning one animal, and nearly non-existent head direction tuning in the third, which could be explained due to slight differences in implant coordinates in the anterior-posterior axis. Since it has been shown that functional units in the prefrontal cortex are clustered in somewhat of a patchwork manner (Le Merre et al., 2021; Gao et al., 2022), it is possible that sampling relatively small volumes of tissue with devices like tetrodes and Neuropixels probes, particularly in cortical areas which extend several millimeters, it is entirely possible to miss small clusters of head direction cells despite looking for them. This hypothesis is supported by the fact that head direction units were identified along most of the dorsal-ventral axis of the probe with pockets of units clustered closer together in some areas. Similar ventral-to-dorsal gradients of encoding were also reported for navigational variables in the PFC of freely moving rhesus monkeys (Maisson et al., 2023), so it would not be unprecedented to find a distributed gradient of functional units within prefrontal areas.

Our data also indicated stronger head direction tuning at farther posterior coordinates,

but the significance of this was not tested in the study. The animal with the strongest directionally-tuned units was also the animal with the most posterior probe location (+2.5mm AP). It was a combination of luck with surgical coordinates, recording probes spanning several millimeters of tissue and detailed, unbiased tracking of the physical features of the animal that account for why we were able to identify this novel tuning feature in the rodent medial prefrontal cortex. What was characteristic of these medial prefrontal head direction units was that the majority of the cells were more broadly tuned than thalamic head direction cells (Jankowski et al., 2014; Ajabi et al., 2023), showing a preference for a general direction in the room and with wider tuning curves. This would fit with a hypothetical role for the medial prefrontal cortex in receiving directional information from upstream areas and integrating it into more abstract representations that can vary with contextual demands. Meaning: I do not need to know whether my head is at a 30° angle or 50° angle relative to a landmark, but I do need to know whether I am heading North or South when navigating towards a goal location. Thus, the initial computations that constitute the neural representations of the behaviors probably happens upstream of the medial prefrontal cortex, with all of the signals being combined to represent the behavioral state of the animal more holistically in medial prefrontal cortex.

5.5 The issue of measuring in traditional and naturalistic experimental designs

I want to finish this discussion by introducing a poem from the 1800s, whose meaning is still highly relevant even today - especially within the field of neuroscience.

"The Blind Man And The Elephant" by John Godfrey Saxe (1816-1887):

It was six men of Indostan, to learning much inclined, who went to see the elephant (Though all of them were blind), that each by observation, might satisfy his mind.

The first approached the elephant, and, happening to fall, against his broad and sturdy side, at once began to bawl: "God bless me! but the elephant, is nothing but a wall!"

The second feeling of the tusk, cried: "Ho! what have we here, so very round and smooth and sharp? To me tis mighty clear, this wonder of an elephant, is very like

a spear!"

The third approached the animal, and, happening to take, the squirming trunk within his hands, "I see," quoth he, the elephant is very like a snake!"

The fourth reached out his eager hand, and felt about the knee: "What most this wondrous beast is like, is mighty plain," quoth he; "Tis clear enough the elephant is very like a tree."

The fifth, who chanced to touch the ear, Said; "E'en the blindest man can tell what this resembles most; Deny the fact who can, This marvel of an elephant, is very like a fan!"

The sixth no sooner had begun, about the beast to grope, than, seizing on the swinging tail, that fell within his scope, "I see," quothe he, "the elephant is very like a rope!"

And so these men of Indostan, disputed loud and long, each in his own opinion, exceeding stiff and strong, Though each was partly in the right, and all were in the wrong!

So, oft in theologic wars, the disputants, I ween, tread on in utter ignorance, of what each other mean, and prate about the elephant, not one of them has seen!

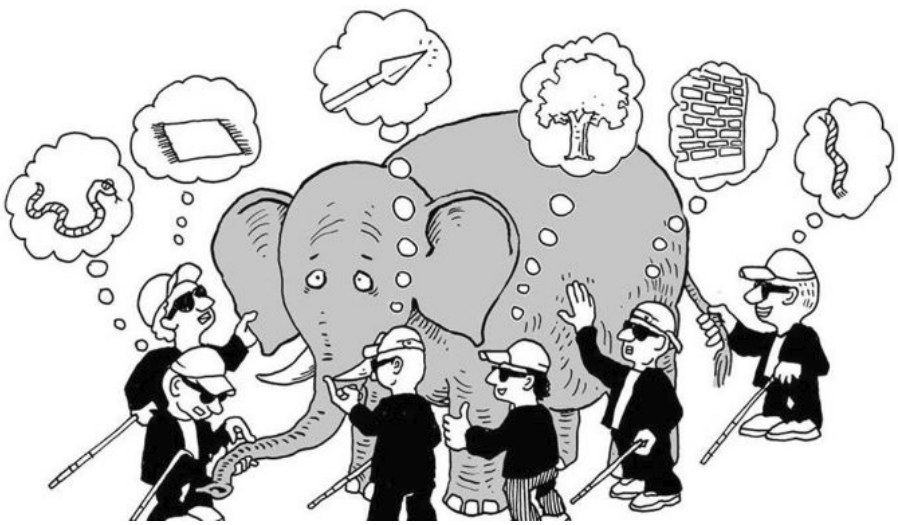

Figure 7. Picture from Jetzek, T. (2016, May 13). The Blind Men and the Elephant: On measuring.

This poem exemplifies how easy it is to misinterpret the data you have, especially if you are investigating individual parts that constitute a whole, without knowing how these individual parts make up this whole, or even if they are a part of a whole at all. A study by Euston & McNaughton (2006) is an example of the importance of being open-minded going into a study and following where the data lead, which in their case resulted in a different conclusion and interpretation of their findings than what might have been the original intent of the study. This is exactly why we chose the three-task paradigm for this study, so as not to be ensnared by the issue of interpreting the data in a particular way without checking the results from a different angle. Using three different tasks with overlapping aspects, we were able to 'triangulate' our findings in an effort to identify "the whole", as what might appear as tuning to a specific feature in one task may be discovered to be a mere correlate, or epiphenomenon, for the actual behavior in another. Naturalistic experimental designs that contain behavioral tasks that are ecologically valid open the door to discovering neural circuits responsible for generating naturalistic behavior. Complementing this approach, traditional task-based paradigms leverage the experimental control that comes with behavioral constraints, and investigate the mechanics of these circuits in greater detail, enabling understanding of the workings of the brain at a different scale. However, this approach also needs naturalistic experiments as a counterpart to understand where and how the circuits serve the behavioral ecology of the organism. We entered into the third study with as unbiased an approach as possible, not pre-defining the time scale of behavior to investigate or which task-variables on which to focus, and rather let the neural data guide our investigations. This helped to avoid the pitfall of using a too-constrained experimental paradigm when measuring behavior (Krakauer et al., 2017; Berman, 2018), and in essence afforded us the opportunity to gaze at the entire elephant, or at least more of it, instead of only grabbing its tail and conclude that we have identified a rope.

Chapter 6

Concluding thoughts

In this thesis, I have shown that there are putative synaptic connections between V1 fibers and M2-projecting neurons in extrastriate areas using 3D reconstructions and high resolution analyses. Additionally I have shown that there is a larger degree of colocalization in layers 2/3, with sparser overlap in deeper layers of the cortex. In my second paper I developed a novel paradigm for observational learning in rats and demonstrated that rats can learn a 2-step behavioral sequence through observation without having to be motivated by deprivation or aversive stimuli. The published paradigm is a proof-of-concept protocol that shows a statistical difference between naive controls and experimental animals, but it can be refined even further with a few tweaks to future iterations. Suggestions are discussed in the text, and I am hopeful that this paper will lay the ground work for future non-fear based studies of observational learning. In the third paper I show that naturalistic behaviors are stably represented in the rodent medial prefrontal cortex at a population level, both for task specific and task general behaviors. I also show that these behaviors cannot be deconstructed into simple pose or movement features, indicating that the medial prefrontal cortex encodes behaviors flexibly rather than rigidly, and at a higher level of abstraction. In the work there are also indications of single units being recruited into behavioral ensembles based on the current behavioral state space of the animal, indicating a flexible coding strategy of recruiting functional units that depends on the current context and goal. This is in line with previous research on isolated functions in the medial prefrontal cortex (Dalley et al., 2004), but here put into a more complete framework that explores how individual units come together, in a population level representation, of naturalistic behavioral patterns. I argue with this work that traditional experimental paradigms that investigate specific prefrontal features in isolation should co-exist alongside more naturalistic designs with freely-moving animals.

This is especially important when investigating a brain area that is involved in the representation and regulation of a multitude of behaviors, and is hypothesized to enable adaptive navigation of dynamic environments and make decisions that are aligned with current goals and intentions of the animal. Thus, we can assume it combines many streams of information that are challenging to isolate properly. To piece together findings from traditional experimental studies, I argue that the animals need to be unrestrained, as evolution intended, so that the brain receives the full complement of signals and feedback it evolved to expect during free movement. In this way the neural recordings carry with them all the information that should come when navigating and switching between situations in everyday life. This is, I believe, how to put puzzle pieces gained with traditional, task-based methodologies together in a naturalistically grounded whole theory of brain function.

Chapter 7

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Paper I

frontiers | Frontiers in Neuroanatomy

TYPE Original Research
PUBLISHED 09 May 2023
DOI 10.3389/fnana.2023.1188808

OPEN ACCESS

EDITED BY
Marcello Rosa,
Monash University, Australia

REVIEWED BY
Daisuke Shimaoka,
Monash University, Australia
Sam Merlin,
Western Sydney University, Australia

*CORRESPONDENCE
Jonathan R. Whitlock
✉ jonathan.whitlock@ntnu.no

RECEIVED 17 March 2023
ACCEPTED 14 April 2023
PUBLISHED 09 May 2023

CITATION
Hovde K, Rautio IV, Hegstad AM, Witter MP and Whitlock JR (2023) Visuomotor interactions in the mouse forebrain mediated by extrastriate cortico-cortical pathways. *Front. Neuroanat.* 17:1188808. doi: 10.3389/fnana.2023.1188808

Visuomotor interactions in the mouse forebrain mediated by extrastriate cortico-cortical pathways

Karoline Hovde[1,2], Ida V. Rautio[1], Andrea M. Hegstad[1], Menno P. Witter[1] and Jonathan R. Whitlock[1]*

[1]Kavli Institute for Systems Neuroscience, Norwegian University of Science and Technology, Trondheim, Norway, [2]Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Introduction: The mammalian visual system can be broadly divided into two functional processing pathways: a dorsal stream supporting visually and spatially guided actions, and a ventral stream enabling object recognition. In rodents, the majority of visual signaling in the dorsal stream is transmitted to frontal motor cortices via extrastriate visual areas surrounding V1, but exactly where and to what extent V1 feeds into motor-projecting visual regions is not well known.

Methods: We employed a dual labeling strategy in male and female mice in which efferent projections from V1 were labeled anterogradely, and motor-projecting neurons in higher visual areas were labeled with retrogradely traveling adeno-associated virus (rAAV-retro) injected in M2. We characterized the labeling in both flattened and coronal sections of dorsal cortex and made high-resolution 3D reconstructions to count putative synaptic contacts in different extrastriate areas.

Results: The most pronounced colocalization V1 output and M2 input occurred in extrastriate areas AM, PM, RL and AL. Neurons in both superficial and deep layers in each project to M2, but high resolution volumetric reconstructions indicated that the majority of putative synaptic contacts from V1 onto M2-projecting neurons occurred in layer 2/3.

Discussion: These findings support the existence of a dorsal processing stream in the mouse visual system, where visual signals reach motor cortex largely via feedforward projections in anteriorly and medially located extrastriate areas.

KEYWORDS

mouse, anterograde tracer injections, retrograde AAV injections, immunohistochemistry, visual cortex, extrastriate, motor cortex

Introduction

A common feature of visual cortical organization across mammals is that visual signals from the eye enter primary visual cortex (V1) via the thalamus, then travel to higher visual areas (Frost and Caviness, 1980; Benevento and Standage, 1982; Simmons et al., 1982; Kaas and Krubitzer, 1991; Salin and Bullier, 1995; Rosa and Krubitzer, 1999) which process progressively distinct components of visual stimuli (Maunsell and Newsome, 1987; Nassi and Callaway, 2009; Niell, 2015; Froudarakis et al., 2019). In primates and carnivores, higher visual processing streams collect into functionally divergent "dorsal" and "ventral" pathways, with the former supporting spatial and visually guided motor behaviors, and the latter

enabling object recognition (Goodale and Milner, 1992; Nassi and Callaway, 2009). Parallel studies in rats, mice and hamsters have also uncovered anatomical (Olavarria et al., 1982; Olavarria and Montero, 1989; Montero, 1993; Wang and Burkhalter, 2007; Wang et al., 2012; Zingg et al., 2014), physiological (Montero and Jian, 1995; Andermann et al., 2011; Marshel et al., 2011; Glickfeld et al., 2013; Garrett et al., 2014; Han et al., 2022) and behavioral (Schneider, 1969; Mlinar and Goodale, 1984; Kolb and Walkey, 1987; Save et al., 1992; Tees, 1999; Ho et al., 2011) evidence supporting a dorsal-*versus*-ventral organization in the rodent visual system, though with fewer functionally specialized nodes. The notion of a dorsal stream in rodents has been further supported by work demonstrating causal contributions of higher visual cortical projections to fine-grained visuomotor control in midline motor cortex in mice (Itokazu et al., 2018), and has prompted investigations into the role of dorsal stream pathways in the production and perception of naturalistic actions (Tombaz et al., 2020; Viaro et al., 2021) and spatial navigation (McNaughton et al., 1989).

Although in mice it has been established that several of the ∼10 (Wang and Burkhalter, 2007; Froudarakis et al., 2019) higher-visual, or extrastriate, areas send direct projections to frontal and midline motor cortices (Wang et al., 2012; Zingg et al., 2014; Itokazu et al., 2018), several pieces of the puzzle remain missing regarding the anatomical chain by which cortical signaling propagates from V1 to motor areas. For example, it is not known whether the output from V1 is uniformly distributed across frontally-projecting extrastriate cortex, if there are regional preferences among these areas or if there is a laminar profile characteristic of such projections.

We sought to address these questions here using a dual pathway tracing approach in which efferent fibers from V1 were labeled using the anterograde tracer 10 KD biotinylated dextran amine (BDA), and motor-projecting extrastriate neurons were labeled via retrogradely traveling, recombinant AAV2/1-retro (rAAV-retro) (Tervo et al., 2016) in secondary motor cortex (M2). Viral injections were targeted to the posterior sector of M2 which receives visual input (Reep et al., 1990; Zingg et al., 2014) and controls movement of the eyes, head and vibrissae (Donoghue and Wise, 1982; Sinnamon and Galer, 1984; Brecht et al., 2004). We visualized the areal overlap of retrograde and anterograde labeling in whole-hemisphere flattened sections of cortex, which revealed a characteristic arrowhead shape of M2-projecting neurons along the anterior perimeter of V1. M2 projecting neurons co-occurred with V1 output fibers in the anteromedial (AM), posteromedial (PM), rostrolateral (RL), and anterolateral (AL) extrastriate areas. In coronal sections, retrograde labeling from M2 spanned superficial and deep layers in anterior extrastriate areas, appearing pillar-like, but became progressively more superficial at farther posterior locations. Morphological 3D reconstructions revealed substantial putative connectivity between V1 axons and M2-projecting neurons in both superficial and deep layers, but with a markedly higher incidence in layer 2/3. Together, our results show that V1 output bound for motor cortex is broadcast non-uniformly across extrastriate regions and is relayed via abundant feedforward projections, particularly in layer 2/3.

Abbreviations: lPPC, lateral posterior parietal cortex; M2, secondary motor cortex; mPPC, medial posterior parietal cortex; P, postrhinal cortex; PPC, posterior parietal cortex; PtP, posterior part of parietal cortex; RSC, retrosplenial cortex; S1B, barrel fields of primary somatosensory cortex; A, anterior area; AL, anterolateral area; AM, anteromedial area; LI, laterointermediate area; LM, lateromedial area; P, posterior area; PM, posteriomedial area; RL, rostrolateral area.

Materials and methods

Eight female C57BL/6JBomTac mice (23–25 g, Taconic) and one male C57BL/6JBomTac mouse (33 g) were used in the project. Five animals received injections of virus and tracer into the left hemisphere and their brains were cut in tangential flattened sections. One brain was excluded from the analysis due to poor uptake of the tracer. Four animals received similar injections in the right hemisphere and the brains were cut in coronal sections. One brain was excluded due to a misplaced injection in V1. All animals were housed in single cages, kept on a reversed day-night cycle, and given *ad libitum* access to food and water. The surgical procedures were approved by the Norwegian Food Safety Authority and the local Animal Welfare Committee of the Norwegian University of Science and Technology and followed the European Communities Council Directive and the Norwegian Animal Welfare Act.

Retrograde viral tracing and anterograde anatomical tracing

For stereotaxic surgeries, the initial coordinates for V1 and M2 injections were calculated in accordance with Paxinos and Franklin (2012) and adjusted based on previous injections in-house. The animals were deeply anesthetized with isoflurane throughout the surgery and their body temperature was kept stable at 37°C. Local anesthetic Marcain (1–3 mg/kg, bupivacaine, AstraZeneca) was injected above the skull, and analgesics Temgesic (0.1 mg/kg, buprenorphine, Indivior, Chesterfield, VA, USA) and Metacam (5 mg/kg, meloxicam, Boehringer Ingelheim, Vetmedica, Germany) were given subcutaneously. After shaving and disinfecting the head (70% ethanol; iodine, NAF Liniment 2%, Norges Apotekerforening), an incision was made along the midline and the skull was cleaned (hydrogen peroxide, H_2O_2; 3%, Norges Apotekerforening), the height of bregma and lambda were measured and adjusted along the anterior-posterior axis to ensure the skull was leveled and two craniotomies were made at the coordinates for injections into secondary motor cortex (M2; AP: + 0.3, ML: + 0.5, DV: −0.5) and primary visual cortex (V1; AP: −4.5, ML: + 2.3, DV: −0.30–0.60) in either the left (N = 5) or right (N = 4) hemisphere. A retrograde GFP-tagged adeno-associated virus rAAV2/1-retro (retrograde AAV-CAG-GFP; serotype "retro," Addgene, Cat. # 37825) was pressure injected into M2 (170, 180, 250, and 400 nL volume injections) by use of glass capillaries [World Precision Instruments (WPI), Cat. No. 4878] and Micro4 pump (WPI; speed 35 μL/s), and the capillary was kept in place for 10 min after the injection, to minimize leakage of the virus. An anterograde tracer, 10 KD biotinylated dextran amine

[BDA, Dextran, Biotin, 10,000 MW, Lysine Fixable (BDA-10,000), Thermo Fisher Scientific Cat. No. D1956, RRID:AB_2307337 in 5% solution in 0.125 M phosphate buffer], was injected into V1 iontophoretically by pulses of positive DC-current (6 s on/off alterations, 6 μA, 10 min) using glass micropipettes (20 μm tip, Harvard apparatus, 30-0044). After the injection was completed, the craniotomies were filled with Venus Diamond Flow (Kulzer, Mitsui chemical group, Cat. # 879566), the skull was cleaned and the skin was sutured and disinfected with iodine. The animal was kept in a heated chamber until awake and active. Post-operative analgesic (Metacam; 5 mg/kg) was given 12 h post-surgery and the health of the animal was closely monitored the days after surgery.

Perfusion and tissue processing

All animals were killed and perfused 21 days post-surgeries.

Tangential flattened sections

The animals that received injections in the left hemisphere were given an overdose of pentobarbital (0.2 mL/100 g) and transcardially perfused using fresh ringer's solution (0.025% KCl, 0.85% NaCl, 0.02% $NaHCO_3$, pH 6.9) and PFA (1%, 0.125 M phosphate buffer, pH 7.4), and the brains were carefully removed and kept in a cup of PFA. Within 1 h, the cortex of the left hemisphere was dissected out and flattened, and tangential sections (50 μm) were prepared. To do so, the intact brain was cut along the midline, subcortical areas and cerebellum were removed, and one cut was made in the fornix dorsal to the anterior commissure. Horizontal cuts were then made along the white matter, and relief cuts were made ventral to postrhinal cortex and in the anterior cingulate cortex. The hippocampus was unfolded, and the cortex was flattened between two microscope glasses covered with parafilm (Laboratory film, Pechiney, Plastic packaging, Chicago, IL, USA) and submerged in PFA (4%) overnight at 4°C with a glass weight on top (52 g). The following day, the flattened cortex was removed from the microscope slides and left in a cryoprotective dimethyl sulfoxide solution (2% dimethyl sulfoxide, DMSO, in 0.125 M phosphate buffer; VWR) overnight. The flattened cortex was then cut in 50 μm tangential sections in one series on a freezing microtome (Microm HM430, Thermo Scientific, Waltham, MA, USA).

Coronal sections

Following the same procedure as above, animals with right hemisphere injections were perfused with fresh ringer's solution and PFA (4%). The brain was placed in a container with PFA (4%) overnight, transferred to cryoprotective solution (DMSO, 2%) and stored overnight. The brain was cut on a freezing microtome in 40 μm sections in three series. The first series was mounted on Superfrost Plus microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany) and used for Nissl staining, the second was processed to reveal the tracer and virus, and the third was stained with 3.3′-Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, USA) against the muscarinic acetyl choline receptor 2 (M2AChR2) or kept as a backup in cryoprotective solution stored at −24°C.

Histology and immunohistochemistry

Nissl

Series one of the coronal sections was stained with Nissl staining. To do so, sections were hydrated in running water and dehydrated in baths with increasing percentage of ethanol (50, 70, 80, 90, and 100% x3), cleared in a solution of xylene (2 min; VWR, International, Fontenay-sous-Bois, France) and rehydrated in decreasing percentage of ethanol, followed by a brief rinse in running water prior to staining in Cresyl violet on a shaker (3 min). The sections were rinsed in water, differentiated in a solution of ethanol/acetic acid (0.5% acetic acid in 70% ethanol; VWR, International, Fontenay-sous-Bois, France) until reaching the desired staining contrast, and cleared in two xylene baths (2 min, 20 min) before being coverslipped with an entellan-xylene solution (Merck KGaA, Darmstadr, Germany).

BDA visualization and enhancement of rAAV-retro signal

All flattened tangential sections and series two of the coronal sections were processed to reveal the BDA tracer and to enhance signal from the virus using a 2-day immunohistochemical procedure. On day one, the sections were washed in phosphate buffered saline (PBS; 3 × 5 min), followed by a phosphate buffered saline solution with Triton (PBS 0.1 M, 0.3% Triton, 3% BSA; 2 × 10 min) on a shaker (100 rpm) at room temperature (RT). The sections were incubated with anti-GFP primary antibody (GFP; rabbit anti-GFP, 1:1,000, Thermo Fisher Scientific, A-11122) overnight on a shaker (60 rpm) at 4°C. On day two, the sections were washed in PBS solution (PBS 0.1 M, 0.3% Triton, 3% BSA; 2 × 5 min) and incubated with secondary antibody (Alexa Fluor 488-tagged goat anti-rabbit Ab, 1:1,000, Thermo Fisher Scientific, A-11008) and with Alexa Fluor 633-conjugated Streptavidin (1:400, Thermo Fisher Scientific Cat. No. S-21375, RRID:AB_2313500) against BDA on a shaker (60 rpm) at RT (75 min). The sections were rinsed in Tris buffer 0.606% [Tris(hydroxymethyl)aminomethane, pH 7.6; 3 × 10 min], mounted on non-frost microscope slides using a Tris-gelatin solution (0.2% gelatin in Tris-buffer, pH 7.6) and coverslipped with an entellan-xylene solution.

DAB staining against M2AChR

Series three of the coronal sections were stained with 3.3′-Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) to visualize M2AChR density and were used only for delineation purposes. To do so, sections were rinsed in PBS (0.125 M, 2 × 5 min) followed by TBS-Tx (2 × 5 min) and incubated with primary antibody (Rat anti-muscarinic M2 monoclonal antibody, unconjugated, clone m2-2-b3, 1:750, Millipore Cat. No. MAB367, RRID:AB_94952; overnight at RT), washed in TBS-Tx (2 × 5 min) and incubated with mouse-absorbed, rabbit-anti-rat secondary antibody [Anti-rat IgG (H + L), 1:300, Vector Laboratories Cat. No. BA-4001, RRID:AB_10015300] for 90 min at RT. The sections were washed in TBS-Tx (2 × 5 min), in PB (2 × 5 min), in H_2O_2-metanol solution (0.08%, Sigma-Aldrich, 2 × 5 min) and in TBS-Tx (2 × 5 min) and incubated with a Vector ABC kit (Vector laboratories, Inc., Burlingame,

CA, USA) for 90 min at RT, per the manufacturer's instructions. They were then washed in TBS-Tx (2 × 5 min) and Tris-buffer (2 × 5 min) before being incubated with DAB (10 mg in 15 mL Tris- buffer, Sigma-Aldrich) at RT. H_2O_2 (2 μL, 30%, Sigma-Aldrich) was added to the DAB solution immediately prior to the incubation. The solution was filtered, the sections were incubated in DAB until the desired level of staining was reached and washed in Tris-buffer solution. A 0.2% gelatin solution was used to mount the sections on Menzel glass slides, the slides were dried overnight on a heated pad and coverslipped with an entellan-xylene solution.

Imaging and analyses

All Nissl and M2AChR stained sections were digitized for analyses using a bright field scanner (Zeiss Axio Scan.Z1). Sections with fluorescence labeling were examined in a fluorescence microscope (Zeiss Axiomager M2) and digitized with a fluorescence scanner (Zeiss Axio Scan.Z1). Lower exposure time was used for the sections with the injection sites in V1 and M2 to avoid saturation of the signal.

High resolution images (63× oil) in z-stacks (typically 70–90 planes, 0.14 μm intervals, 0.05 μm pixel size) were taken of selected sections with fluorescence labeling using a Zeiss confocal microscope (LSM800). The images we deconvoluted in Huygens 19.10 (Scientific Volume Imaging) using the default express deconvolution. The deconvoluted image stack was saved as 16 bit.pic files (one for each fluorescent channel) and opened in Neurolucida360 (MBF Bioscience) for reconstruction.

The outlines of V1 and S1B were drawn on flattened tangential sections using myeloarchitectonic features visible in layer IV (**Supplementary Figure 1**). The same outlines were copied and overlaid on sections cut through superficial layers of cortex (**Figure 1**), where myeloarchitectonic features were not present.

Reconstruction and proximity testing

The deconvoluted image stacks obtained from Huygens 19.10 (see above) were opened and the two fluorescence channels were merged in Neurolucida360. The black point of the image was increased 10%, the white point lowered 90% and gamma was set to 1.20 for visualization purposes, as this enhanced image contrast and removed background noise. Dendrites were traced using the "user-guided tracing" mode with the method "voxel scooping." Specifically, the user traced the dendrites in the image manually with a computer mouse to identify which parts of the image the software would reconstruct, after which spines were detected automatically using the nearest branch mode. The specifications for detecting spines were: outer range = 0.5, Detector sensitivity = 90%, Minimum count = 50, Minimum height = 0.3. The collections of automatically detected spines were subsequently inspected and manually curated. Next, axons were traced manually using the tracing option "direction kernels" and boutons were detected automatically using the nearest branch mode. The typical process width for these methods was 0.77 μm. After the reconstruction was complete, synaptic markers were placed using the "synaptic markers" button with a 0.25 μm requirement and the results were saved as a.DAT file. The file was opened in Neurolucida explorer and a branch structure analysis was performed using the synapses mode and synaptic markers details. The markers with a distance below 0.25 μm were considered as putative synaptic contacts and used in subsequent comparisons between areas. The soma was not reconstructed in Neurolucida but imported as a 2D image into the final 3D reconstruction of neurons for illustration purposes only.

Results

Areal organization of V1 output and M2 input

First, we sought to gain an overview of cortical regions where efferent fibers from V1 and cell bodies of motor-projecting extrastriate neurons were colocalized. To do so, four mice received unilateral injections of BDA targeted to the posterior pole of V1 (**Figure 1**, top panel), which previous work has shown sends projections to all downstream visual areas (Olavarria and Montero, 1989; Wang and Burkhalter, 2007). The same animals received rAAV-retro injections in the posterior sector of secondary motor cortex, M2 [per the nomenclature of Paxinos and Franklin (2012)], due to the high density of visual input it receives in mice and rats (Reep et al., 1990; Wang et al., 2012). 3 weeks after surgery, the brains were removed and the left hemisphere was dissected out, flattened and cut tangentially into sections parallel to the brain surface, allowing us to visualize regional labeling of efferent V1 fibers and M2-projecting neurons in extrastriate cortices. At least seven extrastriate areas were discernable based on the topographical positioning and orientation of projection plexuses relative to V1, with the most prominent labeling from V1 in LM, LI, AL, and PM, with more moderate labeling in RL and AM, and the weakest labeling in area A [**Figure 1**, Top; regional nomenclature per (Olavarria et al., 1982; Wang and Burkhalter, 2007)]. The location of V1 projections to extrastriate areas was consistent across mice, though the relative strength of labeling within regions varied depending on the mediolateral location of the injections in V1 (**Supplementary Figure 1**) and was therefore not quantified here, but can be found in earlier work (Wang and Burkhalter, 2007). The weak labeling in area A could also have been due the injection locations in V1, so it was not analyzed further, despite being an established component of the mouse dorsal visual stream (Wang et al., 2012).

Retrogradely labeled M2-projecting neurons were condensed at the anterior pole of V1, and flanked its medial and lateral borders in a V-shape that in some cases continued as far laterally as area AL, and as far posteriorly as area PM (**Figure 1**, top and **Supplementary Figures 1**, **2**). Regions showing the most extensive coincident labeling from V1 and to M2 were AM, PM and RL, which partly overlapped with posterior parietal cortex (PPC) (Hovde et al., 2018; Gilissen et al., 2021), and sparse labeling of M2-projecting neurons was present area AL. In all extrastriate regions, dual labeling of V1 efferent fibers and M2-projecting neurons was strongest in superficial layers and layer 4, with sparser labeling in deeper layers (**Supplementary Figure 2**), and this pattern was investigated in more detail in coronal sections.

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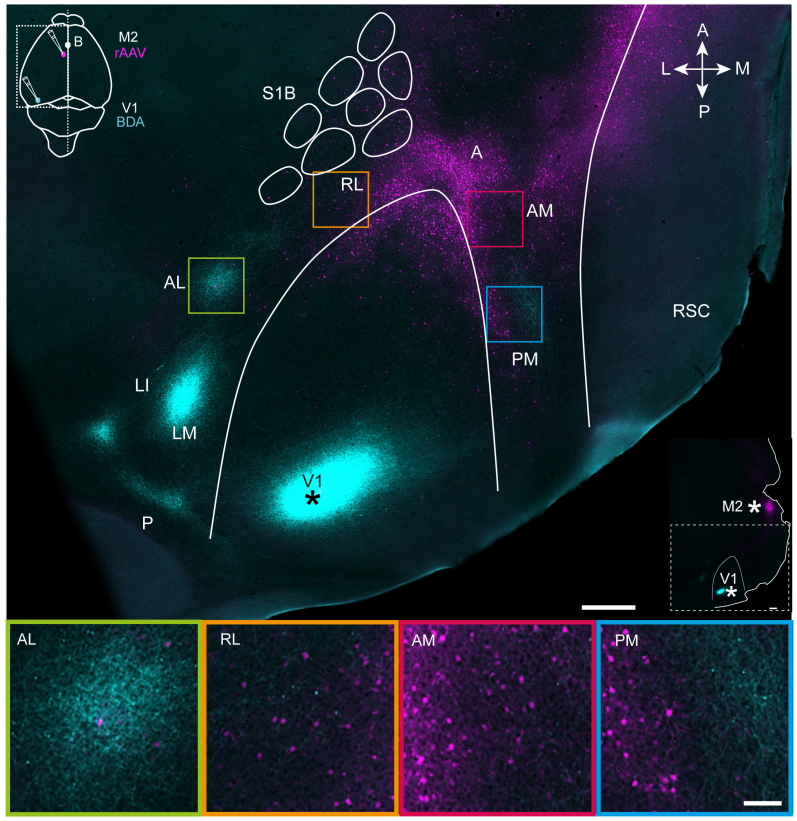

FIGURE 1
Projections from V1 (cyan) and M2-projecting neurons (magenta) viewed in a representative tangential section through superficial layers in flattened dorsal cortex. **Top**, a tangential section through layer 2/3 shows the BDA injection site in V1 (marked with asterisk; see also inset at top left) and projections to extrastriate areas at the periphery of V1. Projection neurons targeting M2 are shown in magenta (injection schematic shown in inset), and colocalized with V1 efferent fibers in areas AL, RL, AM, and PM. The outlines of V1 and the S1 barrel fields were traced using myeloarchitectonic patterns and M2AChR staining from a neighboring section in layer 4 (Methods). Note, a shorter exposure time was applied when scanning the injection site than for projections to avoid signal saturation (Methods). **Bottom**, magnification of extrastriate areas highlighted in the flattened section above. See list for abbreviations. The inset at right indicates injection sites in V1 and M2 with asterisks. White scale bar in upper image = 500 μm; in lower image = 100 μm.

Laminar organization of V1 output fibers and M2-projecting neurons

Similar injections of BDA and rAAV-retro were made in the right hemisphere of V1 and M2 in four additional mice, and coronal sections were collected in cortical regions spanning anteriorly from M2 to the posterior extent of V1 (**Figure 2**, right and **Supplementary Figures 3**, **4**). Consistent with our observations in flattened sections, regions with the densest axonal plexuses from V1 were LM, LI, AL, and PM, with more moderate but clear labeling in AM and RL (**Figure 2**, left and **Supplementary Figures 3**, **4**). V1 projections were observed in both superficial and deep layers of all extrastriate areas, though across animals we noted axonal fibers were concentrated in layers 3 and 5 (**Figure 2**, left, magnifications and **Supplementary Figures 3**, **4**). As with flattened sections, there were very few axonal fibers from V1 in area A.

Across animals, rAAV-retro labeling from M2 was abundant in several extrastriate regions and spanned in a pillar-like fashion from layers 1 to 6 in more anterior regions. At its anterior extent, retrograde M2 labeling formed an apparent pillar at the border of the medial PPC (mPPC) and agranular retrosplenial cortex (RSC; **Figure 2**, 2nd coronal section), then, progressing posteriorly, split into medial and lateral branches that overlapped with mPPC and AM medially, and lateral PPC (lPPC) and RL laterally. Retrograde labeling from M2 was strong in superficial and deep layers in all PPC sub-regions as well as PM and AL as far posterior as Bregma (B) level −3.15 [**Figure 2** and **Supplementary Figures 3, 4, 4th, and 5th** coronal sections; Bregma location based on Paxinos and Franklin (2012)]. Further posteriorly, strong retrograde labeling persisted mostly in superficial layers 1–3 (≥B −3.15) in PM. Laterally, a similar pattern was observed in AL and V1, with the exception that layer 5 was almost devoid of neuronal labeling and layer 6 showed only sparse neuronal labeling (**Figure 2**, 5 and

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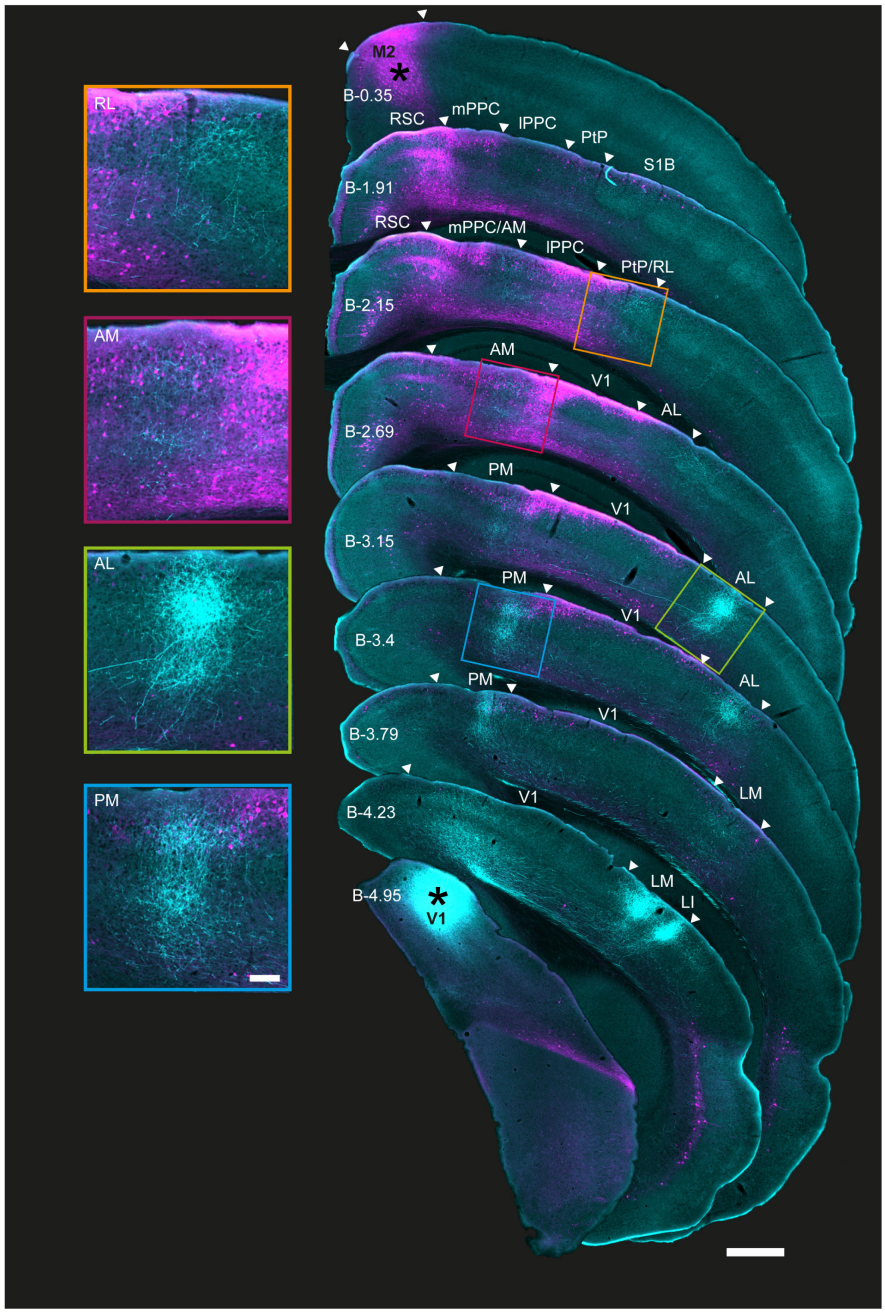

FIGURE 2

Coronal sections showing the laminar profile of BDA-labeled V1 projections and rAAV-retro-labeled M2-projecting neurons in posterior cortices. **Right**, low magnification series of coronal sections from the right hemisphere arranged from anterior (top) to posterior (bottom); injection sites marked with asterisks. Extrastriate areas and PPC boundaries are indicated by white triangles; PPC, its sub-areas, and V1 were delineated using adjacent Nissl and immunohistochemically stained sections from the same series. Anterograde and retrograde labeling from V1 and M2 colocalized in areas RL, AM, and PM, including in PPC, as well as area AL. M2 projecting neurons were found in superficial layers in all regions in the series, and extended to deep layers at more anterior locations, especially in RL and AM. **Left**, magnified view of extrastriate areas highlighted in sections to the right. As with **Figure 1**, shorter exposure times were used for injection sites than projections to avoid signal saturation (Methods); the figure is for illustration purposes. Approximate bregma coordinates (B; Paxinos and Franklin, 2012) are noted on each section; scale bar = 500 μm.

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6th sections; magnified insets, left). Retrograde labeling tapered off completely in all layers at the farthest posterior locations, with no M2-projecting neurons remaining in areas LM or LI, or at the posterior extent of V1 (≥B −4.23; **Figure 2**, right). Thus, of the six identifiable extrastriate areas in this tissue series, regions RL, AM, PM, and AL, in addition to the anterior-most portion of V1, were potential nodes at which visual signals were conveyed to secondary motor cortex.

Neuronal reconstructions and localization of putative synaptic contacts

The colocalization of V1 fibers and M2-projecting neurons in specific extrastriate areas seen here, along with observations from previous studies (Wang et al., 2012; Zingg et al., 2014), suggest these regions serve as nodes for the propagation of visual information to the motor system. However, overlap *per se* does not mean that synaptic connections are in fact present, so we tested this possibility more directly. To do so, we stacked high-magnification (63×) serial confocal scans and created 3-dimensional morphological reconstructions in all extrastriate areas in which M2-projecting neurons had clearly evident pre-synaptic V1 fibers in their vicinity (**Figure 3A**; Methods). Because V1 neurons were labeled by extracellular tracer injections, it is likely that fibers from multiple V1 neurons contributed to each reconstruction. We also note that the resulting reconstructed cells were biased in that they were chosen from areas that had colocalized labeling, which here were AM, AL, PM, and RL, and we do not exclude that similar overlap could occur in area A. Neurons were selected for reconstruction based on three criteria: (i) the presence of a clear and completely filled neuronal soma associated with (ii) filled, long spiny dendrites, and (iii) a sufficiently densely labeled axonal plexus from V1 overlapping the dendrites, where connectivity was expected to occur (see examples in magnified insets in **Figure 3B**). In all sub-areas, neurons meeting these criteria spanned cortical depths ranging from 90 to 640 μm but, due to the preponderance of retrograde labeling from M2 in superficial layers, the majority of reconstructed cells came from layer 2/3. Once sufficiently-labeled cells were identified, putative synapses were identified using close spatial proximity (<0.25 μm) between axonal varicosities and dendritic spines as a proxy for synaptic contacts (Methods) (Wouterlood et al., 2008; Koganezawa et al., 2015), with group data generated for area AM (n = 8 neurons from 3 mice), AL (6 neurons, 2 mice), and PM (7 neurons, 3 mice). Only one mouse had coincident anterograde and retrograde labeling in area RL, so it was excluded from the reconstruction analysis.

Area AM, which had high density retrograde labeling from M2, had the lowest mean number of putative V1 synapses per neuron (**Figure 3B**, top; 38.8 ± 13.7 (mean ± SEM); median = 25; range of 7 to 136 contacts per neuron; all reconstructed AM neurons shown in **Supplementary Figure 5**). Area AL, on the other hand, typically contained few M2-projecting neurons, but had the highest rate of V1 putative contacts of the 3 regions (**Figure 3B**, middle; mean = 263.8 ± 96.8; median = 209.5; range of 23 to 655 per neuron; all reconstructions shown in **Supplementary Figure 6**). In PM, the number of putative synaptic connections from V1 were intermediate between AM and AL across animals (**Figure 3B**, bottom; mean = 81 ± 28.6; median = 71; range of 12 to 183; all neurons shown in **Supplementary Figure 7**). As noted above, the large majority of neurons were reconstructed from superficial layers (n = 6 of 8 neurons in AM; 5 of 6 in AL; 6 of 7 in PM), due to sparse

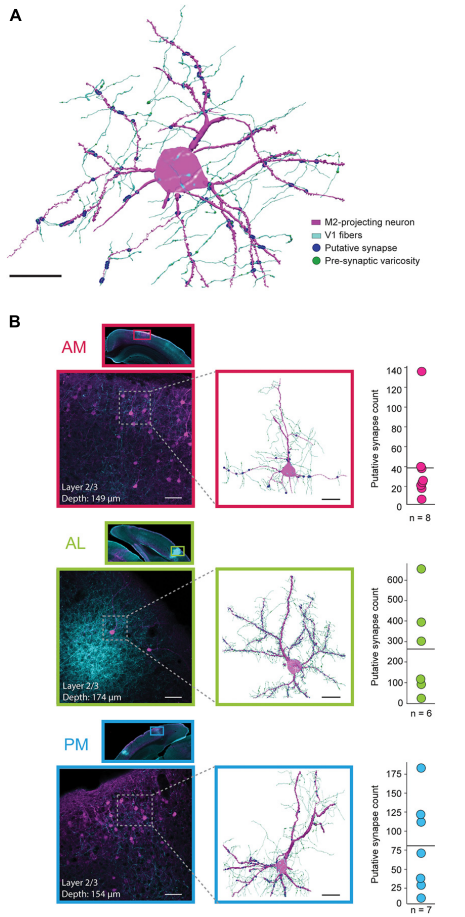

FIGURE 3
Anatomical reconstructions and proximity analysis of V1 axons and M2-projecting neurons in extrastriate areas AM, AL, and PM. **(A)** Representative reconstruction of a single M2-projecing pyramidal neuron from area AL (magenta) receiving synaptic input from V1 axons (cyan); putative synaptic contacts are shown as blue circles and pre-synaptic varicosities are visualized as green enlargements on the V1 axons (varicosities are not visible at points of putative contact due to overlapping blue circles). Black scale bar = 20 μm. **(B)** Top row, inset, a low magnification scan of a coronal tissue section highlighting the portion of area AM where the reconstruction was performed. Top row, lower left, a higher magnification field of view of area AM shown above. Top row, middle, reconstruction of the neuron in the stippled box to the left, with the same labeling convention as the example in panel **(A)**. The depth (from cortical surface to soma center) and cortical layer of each neuron are included in the inset for each example. Top row, right, mean (solid line) and distribution of putative synapses for all reconstructed neurons in area AM. Middle row, same as top, but for as area AL. Bottom row, same as upper rows, but for area PM. Black scale bar = 20 μm. White scale bars = 50 μm.

retrograde labeling and very few incidents of nearby V1 axonal processes in layers 5 and 6 (**Supplementary Figures 5–7**).

Discussion

We used a dual anterograde and retrograde labeling strategy to characterize the regional intersection and connectivity patterns of V1 output fibers onto motor cortical-projecting neurons in mouse extrastriate cortex. We prepared flattened sections, which provided an overview of the entire dorsal cortex, and found that visual and motor pathways overlapped specifically in extrastriate and posterior parietal regions which support visuospatial and motor behavior in rodents (Schneider, 1969; Kolb and Walkey, 1987; McNaughton et al., 1989; Save et al., 1992; Chen et al., 1994; Kolb et al., 1994; Tees, 1999; Nitz, 2006; Harvey et al., 2012; Whitlock et al., 2012; Wilber et al., 2014; Yang et al., 2017; Itokazu et al., 2018). These findings confirm and extend observations in separate studies characterizing efferent projections from V1 (Olavarria et al., 1982; Wang and Burkhalter, 2007) and anterograde projections from extrastriate areas to motor cortices (Wang et al., 2012; Zingg et al., 2014) by directly demonstrating the physical overlap between visual and motor pathways. We additionally generated coronal sections, which revealed pillar-like labeling of M2-projecting neurons from superficial to deep layers in anterior AM and RL, which receded into mainly superficial layers posteriorly in PM, AL and in V1. Higher resolution analyses showed that retrogradely-labeled M2-projecting neurons were more frequent in layer 2/3 than layer 5 and reconstructions of putative synaptic connections showed a trend for higher rates of connectivity in superficial than deep layers.

Though our observations were consistent across animals, there were methodological limitations in the study likely to have influenced the extent of labeling and the resulting patterns of connectivity. In visual cortex, for example, the posterior location of the injection produced the strongest labeling in LM, LI, and AL, whereas area A was only weakly labeled. The high density of anterograde labeling in AL also increased the likelihood of identifying putative synapses, irrespective of the density of retrograde labeling from M2. Furthermore, the medial-lateral location of the injections also likely influenced the distribution of labeling within extrastriate areas (**Supplementary Figures 1, 3, 4**; Wang and Burkhalter, 2007; Hovde et al., 2018), and future work could investigate more systematically whether biases in visuomotor connectivity exist for the medial portion of V1, which subtends the peripheral visual field, and lateral V1, which represents the central, binocular field of view. M2-projecting neurons were also labeled based on only one injection site in the posterior sector, chosen due to its known connectivity with visual regions (Reep et al., 1990; Zingg et al., 2014). However, injecting only in posterior M2 may have led to preferential labeling of more medial extrastriate areas and PPC (Olsen et al., 2019), whereas more anterior injections in M2 would likely have labeled more lateral visual areas subtending different parts of the visual field (Leinweber et al., 2017). The injections themselves in V1 and M2 were also densest in intermediate layers (mainly layers 2–5; **Supplementary Figures 3, 4**), which would favor labeling in matching layers in both up- and down-stream regions (Felleman and Van Essen, 1991; Callaway, 2004). Thus, although we directly observed robust feedforward projections from V1 to extrastriate layers 2/3, projections from V1 to the tips of apical dendrites of layer 5 neurons in layer 1 may have been underrepresented. Because of these constraints, the present study is intended to provide a description of the patterns of labeling in feed-forward visual-to-motor projections, rather than a quantitative account of projection densities or the directionality of connections between visual and motor areas.

Nevertheless, the conjoint use of anterograde and retrograde labeling afforded a direct overview of the anatomical organization of primary visual outputs onto motor cortical inputs in the same preparation. These notably occurred in the same extrastriate regions hypothesized to comprise the dorsal visual processing stream in rodents (Wang et al., 2011, 2012). Intratelencephalic (IT)-to-IT projections, which originate mainly in layers 2/3 and layer 5 (Douglas and Martin, 2004; Harris and Shepherd, 2015), were labeled strongly in our preparations, and the presence of M2-projecting neurons in layers 2/3 and layer 5 suggests that extrastriate and M2 regions participate in mutual feed-forward and feed-back projection pathways (Callaway, 2004; Douglas and Martin, 2004), putting them at nearby stages in the cortical processing hierarchy (Felleman and Van Essen, 1991; Hilgetag et al., 1996). Previous work using dual anterograde tracers showed that regions RL and AM are reciprocally connected with M2, and that all three regions shared directional preferences for visual stimuli and eye movements, suggesting that they comprise an extended sensory-motor network (Itokazu et al., 2018). The frontally projecting neurons we observed in superficial V1 could also participate in mutual feedback loops with frontal motor areas. Based on existing work, such anatomical loops appear to support visual attention (Zhang et al., 2014) and the prediction of expected changes in visual flow due to self-generated movement (Keller et al., 2012; Leinweber et al., 2017).

Although our results provide support for the existence of specialized visual processing streams in rodents, the degree of correspondence with other mammals such as monkeys and carnivores is limited due to considerable differences in brain size, interconnectivity and the relative simplicity of cortical hierarchies in rodents compared to species with more differentiated cortices (Felleman and Van Essen, 1991; Kaas and Krubitzer, 1991; Coogan and Burkhalter, 1993; Krubitzer and Seelke, 2012). For example, whereas visual cortex in primates projects only to nearby higher visual areas and area MT (Felleman and Van Essen, 1991; Hilgetag et al., 2000), V1 in rodents projects to a host of non-visual regions including somatosensory, cingulate, retrosplenial and postrhinal cortices (Vogt and Miller, 1983; Miller and Vogt, 1984; van Groen and Wyss, 1992; Burwell and Amaral, 1998), suggesting a wider and more direct intermixing of visual signals across sensory and cognitive modalities. Thus, visuomotor (Rozzi et al., 2008) and spatial functions (Crowe et al., 2005; Chafee and Crowe, 2012), as well as the reference frames in which they are encoded (Chen et al., 2013), likely follow tighter and more organized anatomical localization in primates and carnivores which, in rodents, appear distributed over coarser topographies. Our present results nevertheless confirm that the anterior and medial extrastriate areas are key sites of visuomotor integration which likely subserve a variety of visually- and spatially guided behaviors in mice.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by the Norwegian Food Safety Authority and the Animal Welfare Committee of the Norwegian University of Science and Technology.

Author contributions

JW, KH, and MW designed the research. KH and AH performed the research. KH, IR, and AH analyzed the data. JW, KH, and IR wrote the manuscript with input from MW and AH. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Research Council of Norway grant N° 300709 (JW), European Research Council ("RAT MIRROR CELL"), starting grant N° 335328; the Centre of Excellence Scheme of the Research Council of Norway (Centre for Neural Computation) grant N° 223262; and the National Infrastructure Scheme of the Research Council of Norway (NORBRAIN) grant N° 197467.

Acknowledgments

We thank M. Andresen, G. M. Olsen, P. Girao, and K. Moan for technical assistance and M. J. Nigro for helpful discussions and comments on the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnana.2023.1188808/full#supplementary-material

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Paper II

Cognitive Neurodynamics
https://doi.org/10.1007/s11571-023-10022-8

BRIEF COMMUNICATION

A novel paradigm for observational learning in rats

Ida V. Rautio[1] · Ella Holt Holmberg[1] · Devika Kurup[1] · Benjamin A. Dunn[1,2] · Jonathan R. Whitlock[1]

Received: 20 February 2023 / Revised: 26 September 2023 / Accepted: 12 October 2023

Abstract
The ability to learn by observing the behavior of others is energy efficient and brings high survival value, making it an important learning tool that has been documented in a myriad of species in the animal kingdom. In the laboratory, rodents have proven useful models for studying different forms of observational learning, however, the most robust learning paradigms typically rely on aversive stimuli, like foot shocks, to drive the social acquisition of fear. Non-fear-based tasks have also been used but they rarely succeed in having observer animals perform a new behavior *de novo*. Consequently, little known regarding the cellular mechanisms supporting non-aversive types of learning, such as visuomotor skill acquisition. To address this we developed a reward-based observational learning paradigm in adult rats, in which observer animals learn to tap lit spheres in a specific sequence by watching skilled demonstrators, with successful trials leading to rewarding intracranial stimulation in both observers and performers. Following three days of observation and a 24-hour delay, observer animals outperformed control animals on several metrics of task performance and efficiency, with a subset of observers demonstrating correct performance immediately when tested. This paradigm thus introduces a novel tool to investigate the neural circuits supporting observational learning and memory for visuomotor behavior, a phenomenon about which little is understood, particularly in rodents.

Keywords Rat · Observational learning · Social · Operant · Brain stimulation · Medial forebrain bundle

Introduction

Throughout life, animals learn new associations and skills through direct experience. However, learning through first-hand interaction demands energy, involves trial, error, and uncertainty, and it can in some cases be dangerous or life-threatening. Observational learning, on the other hand, permits individuals to gain knowledge by interacting with or observing others, often conspecifics, as to which actions to perform, which foods to eat or predators to avoid without risking consequences for themselves. The high survival value of observational learning is reflected by its phylogenetic prevalence, with various forms having been reported in taxa including mammals, birds, reptiles, fish, cephalopods and insects (Russo 1971; Galef 1976; Galef 2005; Zentall 2012; Zentall 2004; Fiorito and Scotto 1992; Laland and Williams 1997; Avargues-Weber and Chittka 2014; Loukola et al. 2017). The diversity and complexity of observational learning imply that many kinds of neural architectures and systems may be involved, and, while many key cellular mechanisms remain unknown, inroads have been made for some types of learning in certain species.

Some of the best progress to date has been made in rodents, which exhibit a variety of forms of observational learning in the laboratory, including acquiring spatial search strategies (Leggio et al. 2003; Yamada and Sakurai 2018), learning novel behaviors to attain food (Russo 1971; Zentall and Levine 1972; Huang et al. 1983; Carlier and Jamon 2006; Jurado-Parras et al. 2012), or forming fearful associations by observing aversive conditioning in conspecifics

Ella Holt Holmberg and Devika Kurup contributed equally to this work.

✉ Ida V. Rautio
ida.v.rautio@ntnu.no

✉ Jonathan R. Whitlock
jonathan.whitlock@ntnu.no

[1] Kavli Institute for Systems Neuroscience, Norwegian University of Science and Technology (NTNU), Olav Kyrresg gate 9, Trondheim 7089, Norway

[2] Department of Mathematical Sciences, Norwegian University of Science and Technology (NTNU), Alfred Getz vei 1, Trondheim 7491, Norway

Published online: 16 November 2023

(Bruchey et al. 2010; Jeon et al. 2010; Twining et al. 2017; Allsop et al. 2018). These and other paradigms often use food restriction, fear or other negative experiences as motivators since they provide strong survival incentives to rapidly acquire conditioned-unconditioned stimulus (CS-US) relationships. Though effective, these approaches also bring limitations. With food deprivation, demonstrators eventually become sated while performing a task, which restricts the number of trials they perform before losing motivation. Fear-based paradigms, though highly effective, rely chiefly on circuits within the amygdala, limbic system and other structures related to affective empathy (Jeon et al. 2010; Kim et al. 2012; Meyza et al. 2017; Allsop et al. 2018; Smith et al. 2021), and are ill-suited for investigating mechanisms of other types of learning, like perceptual-motor translations underlying observational skill learning. Thus, additional approaches are warranted to understand how different forms of observational learning occur in the brain in differing contexts and states.

Here, we present a novel sensory-motor observational learning paradigm that relies neither on food deprivation nor punishment, but instead uses rewarding stimulation to the medial forebrain bundle (MFB) of both demonstrator and observer animals as reinforcement. The task was designed as a form of instrumental conditioning in which an observer animal views a demonstrator tap two internally lit spheres in a particular sequence and, whenever the demonstrator performs a trial correctly (the CS), both animals receive MFB stimulation (the US). Observers viewed performers in this manner for one session per day over three consecutive days, and learning was assessed the following day. The majority of observers acquired the task and significantly outperformed control animals who viewed a similar version of the task with naïve demonstrators. The absence of learning in control animals indicated that observers integrated the actions of skilled demonstrators with contiguous reward to adaptively modify their own behavior, as would be predicted of a successful instrumental conditioning paradigm. These results demonstrate that rats can learn to perform novel behavioral sequences by visual observation, and we discuss ways in which the paradigm can be further refined for future investigations into the neural substrates supporting non-aversive observational learning.

Materials and methods

Animal subjects

All experiments were performed in accordance with the Norwegian Animal Welfare Act, the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the local veterinary authority at the Norwegian University of Science and Technology. All experiments were approved by the Norwegian Food Safety Authority (Mattilsynet; protocol ID # 25094). The study contained no randomization to experimental treatments and no blinding. Sample size (number of animals) was set *a priori* to at least 7 per condition to perform unbiased statistical analyses based on Mead's resource equation. A total of 16 male Long-Evans rats (age: >12 weeks, weight: 393-525 g at the start of experimental sessions) were used in this study. Animals were handled daily from the age of 7 weeks until the start of the experiments. All rats were housed in groups in enriched cages prior to surgery, separated prior to surgery, and housed individually in plexiglass cages (45 × 44 × 30 cm) after surgery to avoid damage to implants. Animals had *ad libitum* access to food and water throughout the entire study and were housed in a temperature and humidity-controlled environment on a reversed 12 h light/12 h dark cycle. Training and experimental sessions all took place during the dark cycle.

Animals from the same litter were paired for behavioral experiments whenever possible, though well-trained performers were re-used for multiple observers in some cases. The roles of "observer" and "performer" were designated randomly if the animals were siblings. In cases where animals were not from the same litter, the older animal was designated "performer". However, we observed no apparent differences in learning effects between sibling and non-sibling pairs.

Surgery

Animals were anesthetized initially with 5% isoflurane vapor mixed with oxygen and maintained at 1–3% throughout the surgical procedure, typically lasting 1–2 h. While anesthetized, they were placed in a stereotaxic frame and injected subcutaneously with Metacam (2.5 mg/kg) and Temgesic (0.05 mg/kg) as general analgesics. A local anesthetic (Marcain 0.5%) was injected under the scalp before the first surgical incision was made, followed by clearing the skull of skin and fascia. Using a high-speed dental drill, multiple holes were drilled in the skull and jeweler's screws were inserted to later anchor dental acrylic (Kulzer GmbH, Germany) at the end of the surgery. A craniotomy was opened over the right hemisphere at AP: -2.8, ML: 1.7, and a single bipolar stimulating electrode targeting the MFB was gradually lowered in the brain to the level of the lateral hypothalamus (DV: 7.8-8.0). Stimulating electrodes were 13 mm long twisted stainless-steel wires coated with polyimide (125 μm diameter, 150 μm with insulation; MS303/3-B/SPC, Plastics One, Canada). Prior to insertion, each electrode was suspended in 70% ethanol for 45–60 min, dried

and rinsed with saline. Once the electrode was in place, the craniotomy was sealed with silicone elastomer (Kwik-Sil, World Precision Instruments Inc., USA), and a thin layer of Super-Bond (Sun Medical Co., Ltd., Japan) was applied to the skull to increase bonding strength between the skull surface and dental acrylic. Once the acrylic was applied and cured, sharp edges were removed with the dental drill at the end of surgery. Following surgery, animals were placed in a heated (32 °C) chamber to recover. Once awake and moving, they were returned to their home cage. If animals showed signs of pain or discomfort post-surgery, additional medication (Metacam (2.5 mg/kg) and Temgesic (0.05 mg/kg)) was given, and antibiotics (Baytril, 25 mg/kg) were administered if wounds showed signs of infection. The testing of stimulating electrodes prior to behavioral training was postponed while animals underwent medical treatment. We noted that the age and weight of the animals were critical factors to consider both in terms of successfully targeting the MFB during surgery, and in the stability of electrode efficacy over time. We used a minimum age of 12 weeks and minimum weight of 300 g as benchmarks, and had the highest rate of surgical success with animals in the weight range of 350 g, +/- 20 g.

Behavioral arena and MFB stimulation set-up

Behavioral experiments were performed in a 93.5 cm x 42 cm x 50 cm clear plexiglass box divided in the middle by a perforated transparent barrier, creating two compartments (Fig. 1). The holes in the barrier allowed for visual, auditory and olfactory cues but were not large enough to allow physical contact between the animals. Two hollow spheres (ping pong balls; STIGA sports AB, Sweden) containing remotely triggered LEDs were mounted on top of hollow metal rods and positioned at the perimeter of the performer's side of the box (Fig. 1a and b). The LEDs in each sphere were connected via insulated wires threaded through the metal rods to a Raspberry Pi 3 (Raspberry Pi Foundation, UK). The Raspberry Pi 3 powered and controlled when LEDs were illuminated, which cued the animals to tap the spheres.

The cue and stimulation schedules in the observational learning experiments were controlled using the Raspberry Pi 3 and a standard personal Dell PC running Windows 10. Custom software written in Python 2 controlled both the LED signaling cues and delivery of electrical brain stimulation during the experiments without experimenter intervention. The Python script also allowed for manual override of the stimulation schedule, if needed, during training of performers (detailed below in Behavioral and Experimental Procedures). All training and experimental sessions lasted 30 min. A Raspberry NoIR Camera V2 (Raspberry Pi Foundation, UK) was turned on each time the script was activated and recorded each session from an overhead view. The experimental room was dimly lit, and 850 nm near-infrared lighting was used to illuminate the video recordings.

Intracranial stimulation of the MFB was delivered via a pulse stimulator (Master 9, Microprobes, USA) and two stimulus isolator units (SIU) (ISO-Flex, Microprobes, USA), each connected to a stimulating cable (305–305 (C)/SPC, Plastics One, Canada) connected to the head of the animal. The size of the implants was kept as small as possible so as not to interfere with the behavior of the animals, and attaching the cable to the implanted electrode had to be done delicately while the animal sat still. The cord from each SIU was attached to a 2-channel commutator (Plastics One, Canada) to prevent the cords from excessive twisting while the animals moved freely in the box. Each stimulation pulse consisted of 500ms trains of square-wave pulses, each lasting 400μsec, with 200μsec on and 200μsec off per cycle, delivered at 150 Hz.

Behavioral and experimental procedures

Habituation

Prior to surgery, experimental animals were handled until calm and comfortable with the experimenter and habituated to the observer-side of the apparatus, where electrodes would eventually be tested. After surgery, but before any intracranial stimulation was given, all animals were habituated to the performer-side of the box on two occasions: once without the stimulating cable attached and a second session with the cable attached. After the efficacy of the electrode was tested in the observer-side of the box (see Electrode testing, below), no further habituation or exposure to the behavioral apparatus was given until the start of the experiments. Prior to the start of any subsequent session, regardless of the stage of training or testing, each animal was allowed to habituate to their compartment until they were calm, either sitting still or grooming. After each session, the box and manipulanda were cleaned thoroughly with detergent (Zalo Ultra, Lilleborg, Norway) and wiped dry to remove trace odors or cues that might influence subsequent experimental or training sessions.

Electrode testing

After a minimum of 5 days of post-operative recovery, stimulating electrodes were tested for efficacy and the strength of stimulation required to reinforce behavior was determined for performers and observers. Efficacy was tested by reinforcing the animal's preference for a neutral object (e.g. a pen) in the observation chamber of the experimental

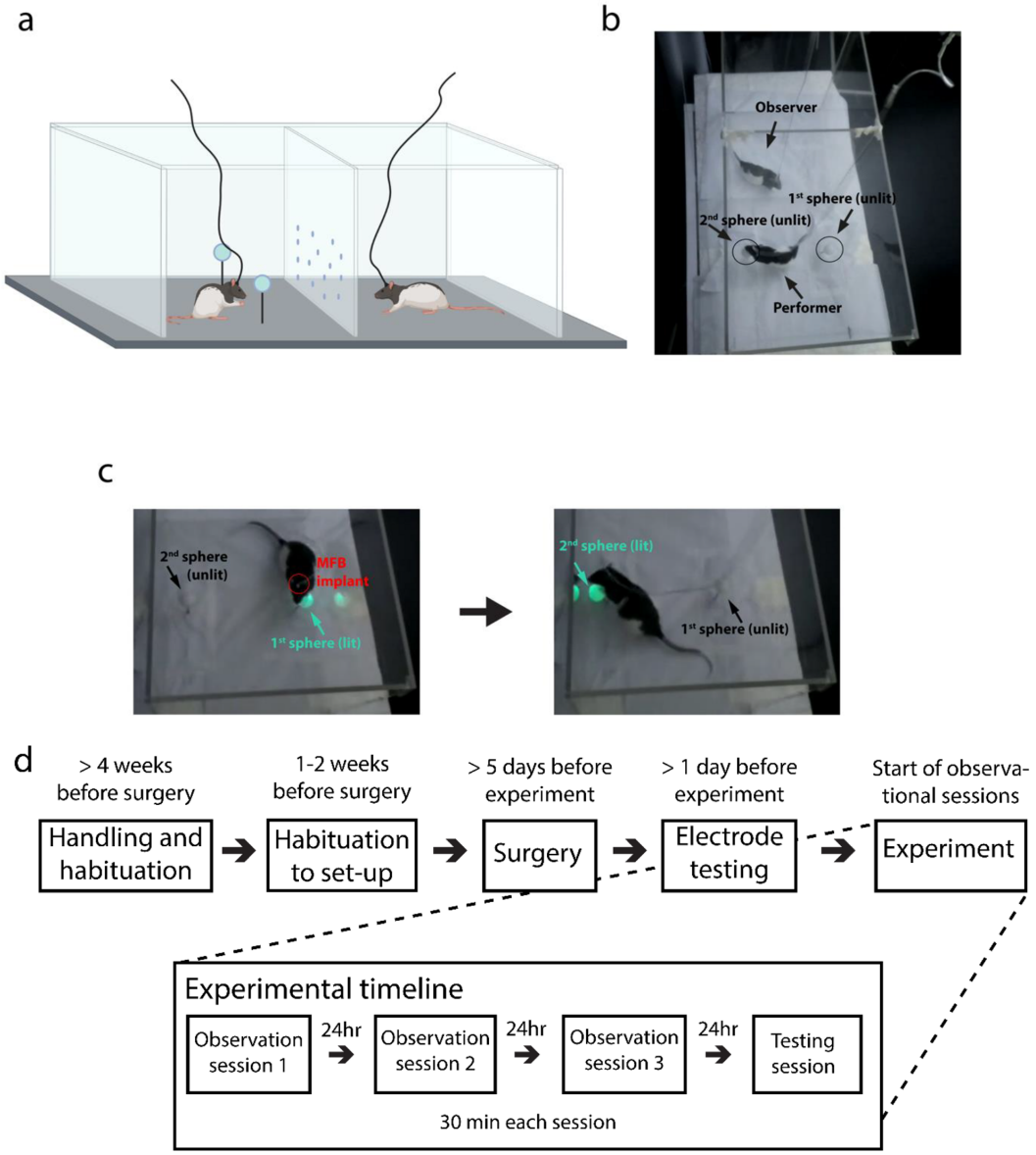

Fig. 1 Apparatus and timeline for behavioral experiments (a) Schematic of the two-sided behavioral training arena, with a demonstrator animal and cue-spheres in the left chamber and an observer in the right. Schematic created with BioRender; illustration not to scale. **(b)** Overhead view of an ongoing experiment, with the performer investigating an unlit cue-sphere (circled), and the observer watching. **(c)** The two-step sequence performed to attain rewarding stimulation. The picture on the left shows a performer tapping the first lit sphere, which triggers the second sphere to light up. The picture on the right shows the moment the animal taps the second sphere and receives intracranial MFB stimulation. **(d)** Total timeline for the experiment, starting from before surgery to after the completion of testing

apparatus. During these tests, the animal was placed in the observation-side of the box and allowed to settle (1–5 min), after which single stimulations were delivered when the animal oriented toward or interacted with the object. All animals started at a stimulation intensity of 20μA. If they were non-responsive then current was increased incrementally by 2μA until behavioral effects were observed, such as increased investigation or physical interaction with the object. Current was then further increased in 2μA increments until side-effects were observed (such as motor artifacts or aversive reactions), or if there had been a cumulative increase of 10μA from the identified effective stimulation intensity without any observed side-effects. If movement artifacts were elicited by the simulation, current strength was incrementally lowered by 2μA until a stimulation intensity that did not elicit artifacts was identified. The final current strength was chosen from the upper range that elicited apparent reward without side-effects (ranging from 18 μA to 60μA across animals). If the optimal range was too narrow or unclear, the animal was re-tested and the optimum was determined on a subsequent day. Electrode testing for performers took place at the start of their training (described below in *Training of performer rats*), and for observers within 24 h before beginning the first observational session.

Training of performer rats

The overall procedure for training demonstrator rats consisted of three phases: (i) an initial shaping phase, followed by (ii) a continuous reinforcement schedule (i.e. stimulation delivered for every sphere-tap), which then changed to (iii) a partial reinforcement phase, in which reward was delivered only when the second sphere was tapped after the first sphere had been tapped without reward. At the start of the first shaping phase, MFB stimulation was given manually whenever the animals oriented toward or approached the spheres to encourage exploration. If no behavioral changes were observed after prolonged bouts of stimulation, or if the animals showed signs of aversion, stimulation current intensity was up- or down-adjusted, respectively. If the stimulation was still aversive or had no noticeable effect, training was discontinued and the rat was excluded from the study. After the animals showed sustained interest in either of the spheres, current intensity was again fine-tuned to the lowest current strength which yielded consistent behavioral responses.

Following initial shaping, subsequent training steps were structured as follows: (1) rewarding stimulation was given when the animal started to physically interact with either sphere, (2) they were rewarded only when starting to tap each sphere alternatingly (partial reinforcement of tapping behavior), (3) rewarded when tapping the spheres as in step 2, but only when the spheres were lit (with lighting controlled manually by the experimenter), (4) withholding reward when tapping the first sphere, but delivering reward when tapping the second sphere when cued by the light, (5) fully automatic training sessions until the animal performed > 75% successful trials per session. Step 4 and step 5 both relied on the Raspberry Pi controlled script to run the task and deliver instantaneous reward after the second tap, but differed in that step 4 allowed for extra motivational stimulation from the experimenter when the performer struggled with the task. Step 5 was initiated only after the animal toggled consistently back and forth between manually lit spheres without additional stimulation from the experimenter. Training was considered complete and stable once a performer exceeded 75% correct trials for three consecutive 30-minute sessions. Learning rates varied from animal to animal, and reaching criterion performance took anywhere from 2 to 15 training sessions. Naïve animals showed no initial spontaneous task-performance and typically required repeated stimulation to acquire the entire task-sequence. Performer rats used for subsequent experimental sessions were given a minimum of one day of rest after reaching criterion. The automatic training sessions utilized the same script as the subsequent experimental sessions.

Experimental task-structure

During the experiments, observer and demonstrator rats were placed in their respective sides of the box and allowed to settle. Once the animals were calm the experimenter initiated the task and an automated script turned on the LED in the first sphere with a random time-interval between 3 and 30 s. The first light remained on for a maximum of 30 s if the demonstrator did not tap the sphere. If > 30 s elapsed, the LED turned off and the trial was scored as a "missed trial", followed by a 3–30 s random time interval (inter-trial interval (ITI)) before the LED turned on again and a new trial started. If the animal tapped the first sphere within the 30 s when the light was on, the first LED turned off and the second LED in the other sphere turned on. The second LED also had a 30 s permissive time window. If the animal tapped the second sphere within this time window, the trial was scored as "successful", and both the performer and observer received concurrent MFB stimulation as a reward. If the demonstrator animal did not tap the second sphere within 30 s, the trial was scored as "failed". After each trial, whether successful or failed, another random ITI of 3–30 s was initiated before the next trial started and followed the same sequence as above. Each session lasted 30 min.

Training and testing of observers

Observer animals observed either a well-trained performer or a naïve control performer (described below in Control group) for one 30-minute session per day over three consecutive days and were tested 24 h after the final observation session. The 24-hour delay was introduced to preclude spontaneous imitation (Zentall 2006). During observational sessions, observers were able to see the performer for the entirety of each 30-minute session while allowed to move freely in their side of the box. On the day of testing, observers were placed in the performer-side of the box, and the same pre-programmed LED- and MFB stimulation-script was run as when demonstrators performed the task. No pre-training or priming with stimulations was given to the observers before testing.

Control group

The control condition was run identically as the real experiments, but with performer animals that were completely naïve to the task. During each of the three control observation sessions, the naïve performer was allowed to move freely on the demonstrator side of the box while a custom script drove the spheres to light up and extinguish in the correct sequence, with reward stimulation delivered to both animals when the LED in the second sphere turned off. The lighting of the spheres and MFB stimulation progressed automatically, irrespective of the demonstrator's actions, and trials were started at random and followed the same randomized 3 to 30 s ITIs as with the experimental trials. This way, reward delivery was dissociated from the behavior of the performer but maintained the sequential structure of correct trials. Critically, this condition also provided the social cue of a would-be performer but lacked the demonstration of task-specific behavior in conjunction with the reward.

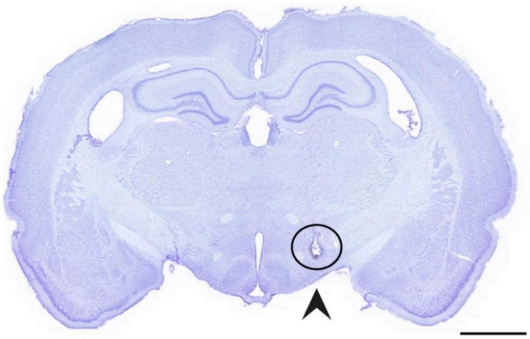

Fig. 2 Histological verification of electrode placement. A Nissl-stained tissue section showing the termination point of a bipolar stimulating electrode targeting the medial forebrain-bundle. Scalebar = 2000 µm

Histology

After experiments were completed, animals were deeply anesthetized with Isofluorane and injected intraperitoneally with an overdose of pentobarbital (Exagon vet., 400 mg/ml, Richter Pharma Ag, Austria), after which they were perfused intracardially with 0.9% saline and 4% paraformaldehyde. The animals were then decapitated, and skin and muscle were removed from the skull before leaving it to post-fix overnight in 4% paraformaldehyde. The following day the electrode implants were removed from the skull and the brains were extracted and stored in dimethyl sulfoxide (DMSO) at 4 °C. On the day of sectioning, the brains were removed from DMSO, frozen with dry ice and sectioned with a sliding microtome at 40 µm in the coronal plane in three series. The first series was mounted immediately on glass slides, Nissl-stained and cover slipped, and the other two series were kept for long term storage in DMSO at -25 °C. Electrode placement was confirmed using the Nissl-stained series (Fig. 2). Of 23 total rats implanted for the final version of this paradigm, two were excluded due to electrodes being off the intended target, and 5 animals had correctly placed electrodes but were excluded due to disruptive behavior (e.g. excessive jumping) on the day of testing.

Results

Observer animals (n = 8) watched skilled demonstrators perform the sphere-tapping task in single 30-minute sessions each day for three days, with demonstrators on average performing 73.7 ± 3.0 (mean ± SEM unless otherwise indicated) trials per session, corresponding to 2.5 ± 0.1 trials per minute, and an overall success rate of 95.8%. A separate group of animals (n = 8) observed three days of the control version of the task, which consisted of naïve demonstrators, the spheres turning on and off in the correct order independently of the demonstrator's behavior, and both observers and demonstrators receiving MFB stimulation whenever the second sphere turned off (average of 87.3 ± 0.8 automated trials per session). Importantly, the total number of MFB stimulations received during the training phase did not differ between experimental and control groups (bootstrapped independent samples t-test, $p = 0.60$). Observers and control animals were tested in the task 24 h after the final training session, with observers performing a significantly higher proportion of correct trials (23.3 ± 8.3%) than controls (5.8 ± 3.1%; Mann-Whitney $U = 14.5$, $p = 0.0325$; Fig. 3). The rate of successful trials varied considerably across observer animals, ranging from just under 60% correct performance in the best animal to 0% in one observer who failed to learn the task (Fig. 3, left). This overall range was intermediate

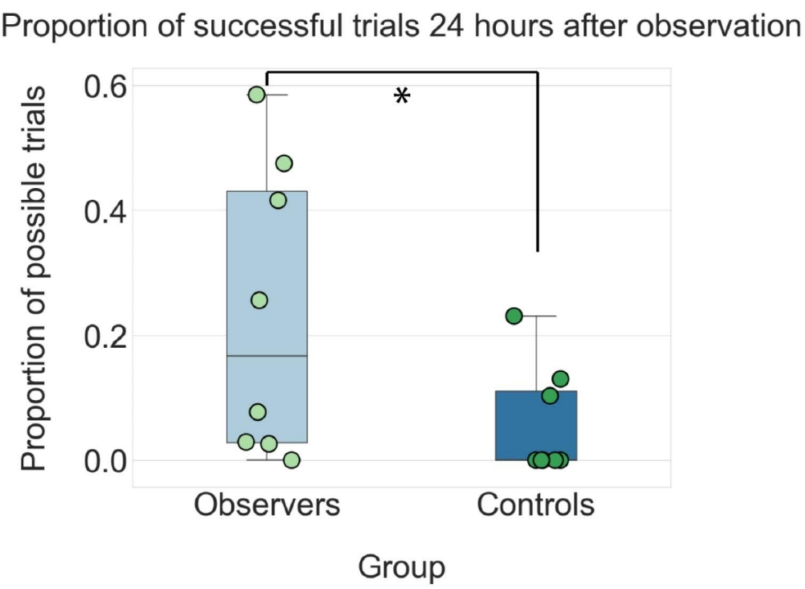

Fig. 3 Observer animals performed a significantly higher fraction of successful trials than controls when tested 24 h after the final observation session. Whisker plots show the variability and overall success rate (out of all trials) for observers (left) and controls (right). Success rates for each animal are shown as individual dots; median performance in each group is indicated by the black line (16.7% for observers; 5.2% for controls); the 25th percentile of the distribution is shown below; 75th percentile is shown above; whiskers show the full distribution of data. Statistical significance between groups ($p < 0.05$; one-tailed Mann-Whitney U test) is noted with a star

between that of first-person trained demonstrators, who achieved 95–100% correct performance after extensive training, and naive animals, who exhibited 0% performance prior to shaping. Compared to observers, the range of performance for control animals was substantially lower, with the best animal performing 23% correct trials and five with 0% (Fig. 3, right).

Observer animals exceeded controls in other aspects of task performance that reflected other features of learning. These included a higher total number of correct trials executed in the test session (mean of 9.3 ± 3.3 for observers vs. 2.3 ± 1.2 for controls; Mann-Whitney $U = 14.5$, $p = 0.0325$; Fig. 4a) and a higher number of trials performed per minute (0.30 ± 0.11 vs. 0.07 ± 0.04 for controls; Mann-Whitney $U = 14.5$, $p = 0.0325$; Fig. 4b), demonstrating higher levels of efficiency and vigor of task execution in trained observers. The faster performance rate of observer animals was also reflected by the shorter average trial length (45.6 ± 3.8 s for observers vs. 54.1 ± 2.6 s for controls; one-tailed Mann-Whitney $U = 50$, $p = 0.0325$; Fig. 4c), and by the fact that the distribution of trial latencies for control animals tended to cluster at the maximal allowable time of 60 s per trial. Perhaps most critically, the mean and median latencies between tapping the first and second sphere were shorter for observers than controls (mean of 20.3 s for observers vs. 25.5 s for controls; Mann-Whitney $U = 14.5$, $p = 0.0325$; median 18.9 s vs. 30.0 s (the maximum), respectively; Fig. 4d), indicating that observers had learned the alternation response between the two spheres rather than approaching and the 2nd sphere directly, as would be expected with stimulus enhancement. The latency to tap the first sphere, by contrast, did not differ between groups (25.5 ± 1.3 s vs. 27.6 ± 0.80 s; one-tailed Mann-Whitney $U = 19$, $p = 0.098$; Fig. 4e), suggesting that the saliency of the lighting of the first sphere was similar at the group level. There were, however, individual differences between observers, with some waiting near the first sphere, tapping it immediately at the start of the trial and turning directly to tap the second sphere. Other observers had longer latencies between the first and second tap and appeared less motivated, as they would tap the first sphere but then explore the chamber or groom before approaching and tapping the second sphere. Finally, we note that several comparisons had the same statistical result (i.e. total successful trials, trials per minute, and mean latency between 1st and 2nd sphere tap) because these aspects of performance were highly correlated, resulting in similar rankings and, consequently, the same U- and p-values.

Discussion

In this study, we demonstrate a new observational learning paradigm in which rats learn to perform a novel sequence of actions by observing the behavior of conspecifics. The task was designed as a means of investigating the neural substrates of observational learning without the use of fear, hunger or other negative experiences as motivators. The task relied on instrumental conditioning principles, where the demonstration of a successful trial by the performer

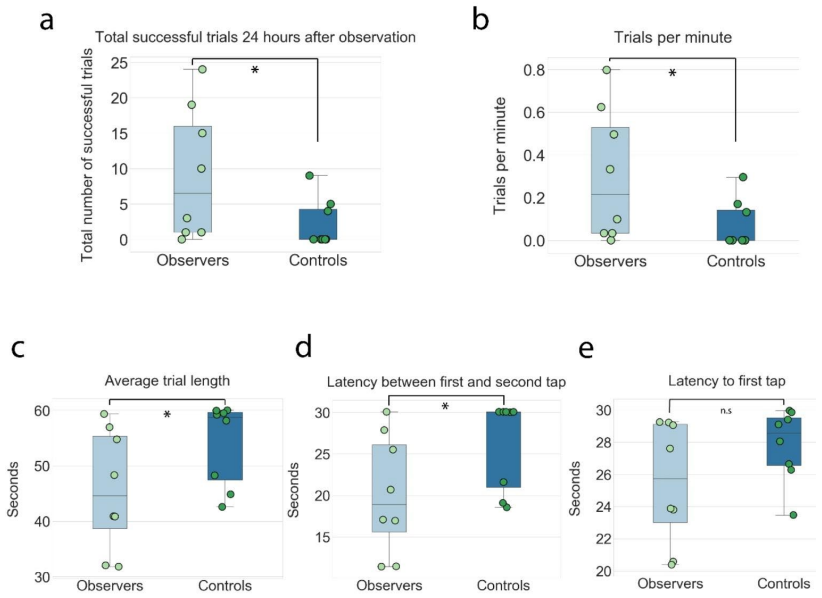

Fig. 4 Observer animals surpassed controls on additional task performance metrics. **(a)** Observers had a higher total number of successful trials than controls during testing; each dot indicates individual animal performance. **(b)** Observers performed significantly more trials per minute than controls, reflecting a higher and more consistent pace of task-performance. **(c)** Observers completed trials significantly faster than controls, measured as the time interval from when first sphere was lit to when the second sphere turned off; maximum possible time for each trial was 60 s. **(d)** Observers also had a shorter average latency to tap the second sphere after the first. The maximum time for each trial was 30 s, with 5 of 8 control animals exceeding this time window. **(e)** The average latency to tap the first sphere after it was lit was lower in observer animals, but the difference was not statistically significant. Significant differences ($p < 0.05$, one-tailed Mann-Whitney U test) are noted with a single star, non-significant differences are noted with n.s.

served as the CS for observers, and rewarding intracranial stimulation served as the US. By undergoing CS-US pairing over three days, observers learned to associate task-specific actions with reward, in effect learning to perform the task by observation. The observer animals performed a higher percentage of correct trials when tested, and they outperformed controls in several temporal metrics indicating a higher efficiency in task performance. Key design features included the use of MFB stimulation to maintain motivation and task engagement for both observers and demonstrators, as well as the use of large spherical cues that were visible from any angle. Crucially, an unrewarded trigger sphere needed to be manipulated first in order to activate the second, rewarded sphere, which required observers to learn the relevant sequence of actions rather going directly for the rewarded sphere or tapping either sphere indiscriminately.

The use of a two-part action sequence was incorporated to reduce the potential contribution of more automatic forms of associative learning, including stimulus enhancement and autoshaping. We argue that stimulus enhancement was not likely the basis for learning since success at the task required the animals to perform a sequence of actions to attain the reward, rather than going straight for the rewarded sphere. Critically, after tapping the first sphere, observer animals tapped the reward sphere more quickly and more often than controls, whereas the latency to tap the unrewarded sphere did not differ, indicating that the cueing stimuli were salient to both groups. Another factor to control for was autoshaping, in which smaller instinctive reactions to a stimulus happen to coincide with a reward and, after some repetition, this leads to the reinforcement of those actions even though they are independent of the reward (Brown and Jenkins 1968). Here, although we cannot rule out a species-specific tendency of the rats to manipulate an illuminated object, autoshaping would more likely come into play if there were only one sphere which needed to be tapped for the reward, rather than first tapping the unreinforced "trigger" sphere. Autoshaping may have been more involved in the initial shaping stages of first-person training, when demonstrators learned that tapping a sphere per se caused rewarding MFB stimulation.

Our broader motivation to create this task stemmed from the fact that much of our current knowledge of the neurobiological mechanisms underlying observational learning derives from fear-based learning paradigms in rodents. Even though fear and pain are powerful stimuli (e.g. Carrillo et al. 2019) which can drive robust learning in different species (John et al. 1968; Olsson et al. 2007; Jeon et al. 2010), observational learning encompasses more than just acquisition of fearful associations, and includes other forms of learning that depend on visual, motor or spatial cognition (Heyes 1994; Galef 2005; Gariepy et al. 2014; Carcea and Froemke 2019). Naturalistic examples have been reported in a wide range of vertebrates and invertebrates, and include acquiring tool usage (Whiten et al. 1999; Biro et al. 2003; Sanz and Morgan 2007; Holzhaider et al. 2010; Loukola et al. 2017), vocal learning (Konishi 2004; Mooney 2014), learning where to shoal or forage for food (Valsecchi et al. 1989a; Laland and Plotkin 1990; Laland and Williams 1997; Emery et al. 2008), or how to solve a specific task to gain access to food (Palameta and Lefebvre 1985; Zohar 1991; Prato Previde and Poli 1996; Carlier and Jamon 2006; Gruber et al. 2009). While remarkable neurobiological insights have also been made, for example, in the songbird learning system (Prather et al. 2008; Vallentin et al. 2016), progress in uncovering the neural bases of non-aversive observational learning has been impeded by a dearth of paradigms which reliably yield *de novo* skill learning in the lab. In rodents, it is more common that observational learning tasks impart a general strategy or prime subjects to perform behaviors (e.g. (Leggio et al. 2003; Jurado-Parras et al. 2012) rather than produce learners who execute a novel behavior from scratch (e.g. (Carlier and Jamon 2006). It was therefore our goal to design a task for rodents that depended on visuomotor cognition in which at least a subset of animals performed the task correctly when first tested.

Another pivotal design aspect of the paradigm was the use of intracranial MFB stimulation as the reward, which brought at least four advantages. One was that we did not need to rely on food or water deprivation, which avoided potential deprivation-related detriments in performance, as well as the loss of motivation from demonstrators once sated. In addition, it avoided the use of traditional aversive motivators, like foot shocks, which was better for the animals' stress and general welfare. The second main advantage, related to the first, was that MFB stimulation was a powerful source of positive reinforcement delivered directly to the brain, bypassing the animals' need for calories or water. It was therefore possible to sustain a high number of trials demonstrated by performers, with an average of more than 70 correct demonstrations in a 30-minute session, and in some sessions > 85. This exceeded the rates of performance for demonstrators in food-motivated operant paradigms (e.g. Carlier and Jamon (2006) reported a total of 70 learning trials summed over 14 daily observational sessions; Jurado-Parras et al. (2012) used a lever-pressing paradigm eliciting ~ 20 trials per 20-minute session) (Valsecchi et al. 1989b). We had previously piloted a version of the present paradigm that depended on food reward and found a steep drop in performance by demonstrators after ~ 10 min, presumably due to the animals reaching satiety. A third advantage of MFB stimulation was the consistent salience of the reward, which may have helped sustain task engagement for observers as well as performers. Previous studies also sought to enhance the attention of observers by using performers of the opposite sex (Collins 1988; Carlier and Jamon 2006), or by tapping into the natural inclination of weanling animals to observe their mothers or older adults (Valsecchi et al. 1989a; Prato Previde and Poli 1996). The use of weanling animals in particular proved effective, though using pups would present challenges for recording or manipulating the brain due to their small size. A final advantage of using MFB stimulation was that its delivery, as well as the other timed features of the task, were automated, giving precise programmatic control over the timing of events within a trial. This reduced training variability within and between animals and allowed us to systematically adjust timing intervals (e.g. upper and lower bounds of inter-trial intervals) while developing the task.

As can be seen from the wide distribution of performance rates among observers, a majority of trained observers learned the task while others apparently did not, so there is still room for improving the paradigm. Based on earlier in-house observations, one way to better the performance of observers would be to increase the total number of observation trials by increasing from three to 5 days of training. For example, while developing the task, two pilot animals with 5 days of training gave > 91% successful performance during testing. However, a caveat was that the animals were tested immediately after the final observational session, so their high rate of performance could have been explained as a result of spontaneous imitation (Zentall 2006). This prompted the inclusion of a 24-hour delay to remove such effects, though we note that reducing the delay to 8 or 12 h might produce more robust performance while still testing genuine associative learning. Another way to increase the total number of observation trials would be to use session lengths longer than 30 min, but careful attention should be given not to fatigue the demonstrator animals. We found that well-trained performers were in constant motion for over 30 min, and most animals' performance began to decline beyond this point.

In summary, although the present study had a limited sample size and the learning effects varied across observers, it demonstrates the proof of principle for an observational

learning paradigm in rats which does not require aversive stimulation or food deprivation. With further refinement, the task could serve as a powerful tool for studies seeking to elucidate the neural substrates supporting the acquisition and long-term (> 24 h) memory of non-aversive observational learning. More generally, the establishment of such a paradigm opens the door for a broader comparison of pathway-specific processes supporting different kinds of observational learning, such as those depending on visuomotor cognition *versus* fear-motivated associative learning.

Acknowledgements We thank M. Andresen, C. Bjørkli, K. Haugen, A.M. Amundsgård, P. J. B. Girão, and H. Waade for technical and IT assistance; S. Eggen for veterinary oversight; M. P. Witter, G.M Olsen, K. Hovde and members of the Whitlock lab for helpful discussions.

Author contributions Conceptualization: Jonathan Whitlock, Benjamin Dunn, Ida V. Rautio; Methodology: Ida V. Rautio, Benjamin Dunn, Jonathan Whitlock; Formal analysis and investigation: Ida V. Rautio, Ella Holt Holmberg, Devika Kurup; Project administration: Ida V. Rautio, Jonathan Whitlock; Funding acquisition: Jonathan Whitlock; Resources: Jonathan Whitlock; Supervision: Jonathan Whitlock, Benjamin Dunn, Ida V. Rautio; Writing - original draft preparation: Ida V. Rautio; Writing - review and editing: Ida V. Rautio, Jonathan Whitlock, Benjamin Dunn, Ella Holt Holmberg, Devika Kurup.

Funding This work was supported by a Research Council of Norway FRIPRO grant (No. 300709) to J.R.W., an NTNU Medical Faculty Fellowship (RSO) to I.V.R., the Centre of Excellence scheme of the Research Council of Norway (Centre for Neural Computation, grant No. 223262), the National Infrastructure scheme of the Research Council of Norway – NORBRAIN (grant No.197467), and The Kavli Foundation.
Open access funding provided by NTNU Norwegian University of Science and Technology (incl St. Olavs Hospital - Trondheim University Hospital)

Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Compliance with ethical standards The authors declare that they have no conflict of interest.

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Paper III

This paper will be submitted for publication and is therefore not included.

Behavior-state representation in the rat medial prefrontal cortex

Ida V. Rautio[1], Fredrik Nevjen[2], Ingeborg Hem[2], Benjamin A. Dunn[2*] & Jonathan R. Whitlock[1*]

[1]Kavli Institute for Systems Neuroscience, Norwegian University of Science and Technology (NTNU), Olav Kyrres gate 9, 7089 Trondheim, Norway

[2]Department of Mathematical Sciences, Norwegian University of Science and Technology (NTNU), Alfred Getz vei 1, 7491 Trondheim, Norway

* These authors jointly supervised the work done in this study.

Acknowledgements

We thank M. Andresen, K. Haugen, A.M. Amundsgård, P. J. B. Girão, and H. Waade for technical and IT assistance; S. Eggen for veterinary oversight; M. P. Witter, G.M Olsen, David Klindt and current and alumni members of the Whitlock and Moser lab for helpful discussions.

Funding

This work was supported by a Research Council of Norway FRIPRO grant (No. 300709) to J.R.W., a Research Council of Norway grant (iMOD, No. 325114) to B.A.D., an NTNU Medical Faculty Fellowship (RSO) to I.V.R., the Centre of Excellence scheme of the Research Council of Norway (Centre for Neural Computation, grant No. 223262), the National Infrastructure scheme of the Research Council of Norway – NORBRAIN (grant No.197467), and The Kavli Foundation.

Compliance with Ethical Standards

The authors declare that they have no conflict of interest.

ISBN 978-82-326-7636-1 (printed ver.)
ISBN 978-82-326-7635-4 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

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