Modularity of Feedback for State-Dependent Guidance of Navigation in Mouse Visual Cortex

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Modularity of Feedback for State-Dependent Guidance of Navigation in Mouse Visual Cortex
by
Andrew Meier

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

January 2021
St. Louis, Missouri
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Acknowledgments

The research described here was made possible by the excellent mentorship and support of my thesis advisor, Dr. Andreas Burkhalter. Thanks to current and former members of the Burkhalter Lab for creating a stimulating scientific environment and for providing technical training and consultation. Thanks to all members of my thesis committee for important feedback throughout the development of these projects. Dr. Ed Han and members of his lab were instrumental in the 2-photon imaging portion of this research, as they provided me with training in calcium imaging and analysis, as well as custom analysis software. Dr. Tim Holy and members of the Holy Lab provided valuable consultation and training throughout my graduate career. Thanks also to Dr. Quanxin Wang and Jehan Ganachaud for collaborating on the project described in Chapter 2. Special gratitude goes to Sally Vogt, who has consistently supported me and fellow graduate students as Neuroscience Program Coordinator. Thanks to Katia Valkova and Dr. Weiqing Ji for great lab management, maintaining mouse lines, and making everything else in lab ran smoothly. Funding for this research was provided by National Eye Institute grants RO1 EY16184, RO1 EY022090, RO1 EY027383 and the McDonnell Center for Systems Neuroscience.

Special thanks to the Washington University Graduate School for permission to use their dissertation templates for guidance in writing this document.

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January 2021

v
Navigating through unpredictable environments requires an efficiently organized sensory system capable of adapting to behavioral demands. In the mammalian visual system, two adaptations which have arisen to meet these demands are parallel processing and top-down feedback of internally generated expectations and contextual information for adjusting responses to match the needs of the current behavioral task. Mouse visual cortex exhibits the latter of these organizing principles, like other mammals, in the form of a molecular layer (Layer 1) which receives feedback to contextually adapt sensory responses from the outside world. The first of these organizing principles, parallel processing streams, generally takes the form in the visual cortex of interdigitating modules with distinct connectivity and physiology. Modular organization clusters similarly tuned cells, improving the signal-to-noise ratio, while minimizing total axonal wire length\(^1,2\). Early studies on mouse visual cortex were inconclusive, however, in finding anatomical or physiological clustering representing parallel processing modules\(^3\). Subsequent research revealed that mouse visual cortex is in fact modularly organized: Layer 1 contains regularly spaced patches of high and low muscarinic acetylcholine receptor 2 expression (M2+ patches and M2- interpatches). Cells aligned with M2+ patches are tuned for shape while those aligned
with M2- interpatches are tuned for features of stimulus motion. In this thesis I further explored M2 modularity by investigating whether M2+ patches and M2- interpatches show specializations for visual guidance of particular behavioral tasks. In the first study, I address this question anatomically by demonstrating that mouse higher visual cortex is modularly organized, comprising interspersed, parallel input and output streams to brain systems responsible for visual processing, landmark identification, and fear regulation. I use pathway tracing and retinotopic mapping to show that the areas exhibiting this modularity reside within the visual ventral stream and establish that the strongest outputs to the amygdala emerge from an anatomically distinct region, the postrhinal area. In the second study, I investigate the relationship between M2 modules and locomotion, which increases the response gain of visual cortex neurons. By using calcium recordings in awake mice during locomotion and visual stimulation, I find that M2-interpatches are more responsive to locomotion and that locomotion increases long-range interpatch-interpatch correlations, allowing for integration across the visual field. I show anatomical organizations which may underlie this physiology, in increased inputs from somatostatin interneurons to M2- interpatches. Finally, I show that M2- interpatches have specific connectivity with the primary and secondary motor cortex. Together, these studies demonstrate that visual cortex is organized into modular subnetworks with distinct specializations for top-down visual guidance of behavior.
Chapter 1: Introduction

The earliest models of visual cortex described a system of feedforward inputs combining sequentially to form complex representations of objects in the environment\(^4,5\). Subsequent findings revealed the necessity of two conceptual additions: spatially separated, modular processing streams\(^6,7\) and feedback projections which rapidly tune sensory responses to meet the needs of changing behavioral goals\(^8,9\). While these features of functional architecture were originally explicated in more visually specialized animals, such as cat and primate, both have also recently been found in mouse visual cortex. In this thesis, I explore these organizational principles by investigating the anatomy and physiology of M2+ patches and M2- interpatches.

In Chapter 1, I will first discuss the concept of modular organization in sensory processing and its manifestation in mouse visual cortex in form of M2+ patches and M2- interpatches. Next I will describe the visual postrhinal cortex (POR) and its role in processing affective and navigational information, as a promising cortical area in which to study modular organization. Lastly I will review locomotion modulation of visual responses and how this phenomenon may be used to determine the role of modularity in the visual guidance of behavior. Chapter 2 will describe a study of modularity in POR and the connectivity of higher visual cortex with the amygdala, visual thalamus, and entorhinal cortex. Chapter 3 describes a physiological study of locomotion modulation in M2+ patches and M2- interpatches, followed by an anatomical investigation of the architecture underlying this behavioral state-dependence of visual responses. In Chapter 4, I draw general conclusions from the results of these experiments, discuss possible
functions of modular organization across visual cortical areas, and pose potential follow-up studies to better understand the role of M2 modularity in visually guided behavior.

1.0 CONTRIBUTION AND COPYRIGHT INFORMATION

Chapters 2 and 3 contain studies which were designed in collaboration with my thesis advisor, Dr. Andreas Burkhalter. Chapter 2 is a manuscript currently in revision for publication in the *Journal of Neuroscience*. Data in these experiments were collected by myself, Dr. Burkhalter, Dr. Quanxin Wang (former member of the Burkhalter Lab now at the Allen Institute for Brain Science), Dr. Weiqing Ji, and Jehan Ganachaud. Chapter 3 consists of a manuscript currently being prepared for submission to a peer-reviewed journal. Physiology data in these experiments were collected by myself in collaboration with Dr. Ed Han. Anatomical data were collected by myself, Dr. Burkhalter, Dr. Weiqing Ji, Dr. Pawan Bista, and Dr. Rinaldo D’Souza. I performed all analyses of this data.

1.1 MODULARITY IN MOUSE VISUAL CORTEX

The classical model of feedforward visual processing involves luminance contrast signals being transduced and processed in the retina, relayed to the dorsal lateral geniculate nucleus (dLGN), then sent to primary visual cortex, where distributed hierarchical processing results in perception of salient visual objects. Retinal signals are also sent along a processing stream important for orienting and spatial attention which passes through the superior colliculus and lateral posterior thalamic nucleus (LP), eventually targeting visual cortex, especially extrastriate areas and L1 of V1. The retina→dLGN→V1 pathway is not a single homogeneous stream, as each stage includes parallel but interconnected processing streams specialized for extracting particular
features from the stimulus. In mouse, this includes multiple retinal ganglion cell types\textsuperscript{15} and the shell and core of the dLGN, which preferentially target L1 or L4 of V1, respectively\textsuperscript{16,17}. Early research on mouse visual cortex, however, did not find clear signs of modularity, as tuning for stimulus features (e.g. orientation) appeared to exhibit ‘salt and pepper’ organization\textsuperscript{3}.

Subsequent studies found that while mouse visual cortex does not contain the same modular organization as more visually specialized animals, such as cytochrome oxidase blobs\textsuperscript{7} or orientation preference pinwheels\textsuperscript{18}, it does however exhibit some forms of clustering and columnar organization. For example, shared orientation preference exists in some minicolumns of cell bodies and clusters of apical dendritic branches contained within approximately 20µm-wide columns share orientation tuning\textsuperscript{19,20}. In deeper layers, L5 projection neurons within a column share long-range projection targets, orientation tuning, and ocular dominance\textsuperscript{21,22}.

This thesis is focused on a related form of modularity, that of muscarinic acetylcholine receptor 2 (M2) clusters. Two recent studies demonstrated a pattern in L1 of interdigitating ~60µm-wide modules with high and low M2 expression (M2+ patches and M2- interpatches) and center-to-center spacing of ~120µm\textsuperscript{23,24}. L2/3 cells aligned with M2+ patches receive preferential L1 inputs from the dLGN shell\textsuperscript{16} and extrastriate areas and have stronger orientation and spatial frequency tuning, suggesting a role in processing shape. M2- interpatch-aligned pyramidal cells receive L1 inputs from the lateral posterior nucleus (LP, i.e. pulvinar) of the thalamus, have stronger tuning for motion coherence and speed, and receive stronger inhibition from parvalbumin-positive interneurons\textsuperscript{23,25}. These features suggest that M2- interpatch cells are specialized for detecting motion, including that of unexpected objects\textsuperscript{17}. M2 modularity is also
present in rat V1$^{23}$ and may align with the ‘honeycomb’ organization described in rat V1$^{26}$, with M2+ patches corresponding to thalamo-recipient zinc-negative hollows and M2- interpatches corresponding to zinc-rich walls.

An important distinguishing feature of M2 modularity in mouse cortex is that M2 modules are found in L1, rather than L2-6, where the (possibly homologous) cytochrome oxidase blobs are found in primate in an interdigitating pattern with M2+ patches in V1$^{6,23}$. Unlike L2-6, L1 contains no excitatory projection neurons and only a sparse collection of interneurons$^{27,28}$, and instead consists predominantly of distal tuft dendrites from pyramidal neurons in lower layers and long-range inputs to these dendrites$^{29-31}$. These inputs integrate internally generated expectation and contextual information with feedforward stimuli arriving at basal dendrites in lower layers$^{32,33}$. This architecture therefore suggests that M2 modularity in mouse plays a role in segregating streams of top-down input which modify the responses of underlying cells to adapt to different contexts.

In the studies described in Chapters 2-3, I address the question of how M2 modules relate to top-down feedback through two lines of investigation. In Chapter 2, I trace the connectivity of M2 modules in the higher visual area POR$^{34}$ with areas that drive behavior. M2 modules were previously only explored in V1, a low-level area in the visual hierarchy, therefore focusing on POR, a higher-order area more closely connected with regulators of affective and navigation behavior, provides deeper insight into the relationship between modularity and contextually responsive feedback inputs. In Chapter 3, I record neural activity in awake behaving mice, and compare how behavioral state – locomotion vs. rest - modulates visual responses in M2+ patch
and M2-interpatch cells. This experiment directly tests whether contextual modulation of responses differs between visual cortex modules, and follow-up anatomical experiments elucidate the architecture supporting these physiological differences.

1.2 POSTRHINAL CORTEX

POR is a homolog of parahippocampal cortex in primate, which is associated with visual scene processing and provides projections to the amygdala\textsuperscript{35–37}. POR has been defined in rodents as lying posterior to the perirhinal and ectorhinal cortical areas and ventral to area TEp (posterior temporal area), a higher visual area in temporal cortex\textsuperscript{38,39}. POR underlies two forms of higher-order visual processing.

First, POR integrates visual inputs to extract navigational information, forming the basis for higher-level spatial representations downstream in the medial entorhinal cortex (ENTm)\textsuperscript{12,40–42}. The activity of POR neurons tracks important variables used in spatial navigation, including running speed\textsuperscript{43}, heading\textsuperscript{40}, location within the environment\textsuperscript{42,44}, and physical boundaries\textsuperscript{45}. In this role, POR comprises the primary route through which spatial location and contextual cues are conveyed to ENTm and the hippocampus\textsuperscript{46}. This pathway is often contrasted with the pathway carrying nonspatial information to the hippocampus through lateral entorhinal cortex via perirhinal cortex\textsuperscript{47,48}, a multimodal association area\textsuperscript{46}. POR’s role in spatial processing has been demonstrated across species with markedly different organizations of visual cortex; for example, the human homolog of POR is necessary for performance at navigation tasks in virtual reality environments\textsuperscript{49}. 
The second major function of POR is the association of visual objects with emotion, especially reward, via connections with the amygdala. The POR-amygdala circuit also carries negative affective associations, as lesions to POR impair expression of fear memories associated with visual stimuli and contexts. POR→amygdala projections are part of the ‘cortical route’ for visual fear responses, supporting complex but higher latency aversive associations than the ‘subcortical route,’ which travels through the superior colliculus, lateral posterior nucleus (LP), and other subcortical nuclei before terminating at the lateral amygdala (LA).

The two major functions of POR depend on input from subcortical nuclei. The tuning for navigational cues displayed in POR cells suggests dependence on inputs from the visual thalamic nuclei, of which POR is most strongly connected to the LP. LP tunes visual responses of cells in POR and the extrastriate cortex by increasing selectivity for fast-moving stimuli, which are essential navigational cues during self-motion. During locomotion, the responses of LP axon terminals in V1 are distinct from projections from the dorsal lateral geniculate nucleus (dLGN) in that LP signals unexpected motion that is incongruent with the mouse’s running speed, whereas dLGN inputs signal congruent flow stimuli. These locomotion-related signals may be integrated to form the distance-tracking receptive fields recently found in POR neurons. Visual thalamic inputs tuned for egocentric location and orientation may also support the central role of POR in identification of spatial contexts.

The second function of POR, its association of visual stimuli with affective responses, depends on its connectivity with the amygdala, particularly LA (lateral amygdala). LA serves as a point of convergence for threat- and reward-related information, projecting to the basolateral nucleus and
central nucleus of the amygdala to drive behaviors such as flight, freezing, and approach\textsuperscript{65,66}. The amygdala also provides feedback to the visual cortex. Feedback projections from higher cortical areas, as well as subcortical structures such as LA, predominantly target L1, synapsing on apical dendrites of pyramidal cells with somas located in deeper layers\textsuperscript{24,67,68}. Feedback from LA to POR has been shown to control hunger-dependent responses to conditioned visual stimuli\textsuperscript{50}. Projections from amygdala to cortical L1 likewise are necessary for retrieval of auditory fear conditioning\textsuperscript{68}.

This dual functionality of POR, processing affective and visuospatial information, is underscored by the finding that most cells within POR are tuned either for visual object identity or for the emotional salience of visual stimuli\textsuperscript{52}. This separation was demonstrated by Ramesh et al. (2018), in an experiment which paired food reward with a particular stimulus orientation, then reversed the orientation-reward pairing after multiple weeks of training, and tracked whether cells maintained tuning for orientation or instead became responsive to the new reward-paired stimulus. ‘Predicted outcome’ neurons were active in response to reward-associated stimuli, switching their preferred stimulus after reversal to match the new food-predicting stimulus. ‘Identity’ neurons instead were responsive to a preferred orientation of the grating stimuli that were presented, maintaining their orientation preference regardless of its association or lack of association with reward. Similar results were found by Sugden et al. (2020)\textsuperscript{53}, who showed that food reward-tuned cells were mostly unresponsive to visual stimuli which did not connote reward. This study also demonstrated the importance of feedback inputs for the affective processing function of POR, by showing that consolidation of stimulus-reward pairings required post-exposure activation of neural ensembles, when feedforward inputs are no longer active.
The presence of these separate populations raised the question of whether POR exhibits spatial clustering corresponding to these two functions. These two functions of POR also suggested that projections from its two major subcortical sources of modulatory input, the amygdala and visual thalamus, may be segregated. This organization would align with previous experiments which demonstrated that amygdala→POR and LP→POR projections only target a subset of cells within POR\textsuperscript{50,69}, similar to the segregation of subcortical inputs to M2 modules within V1\textsuperscript{23,25}. In Chapter 2, I address both of these possibilities by tracing projections from dLGN, LP, and LA to M2+ patches and M2- interpatches in POR. I then use retrograde tracing to determine whether the outputs of POR to its two primary targets, LA and ENTm, are associated with a specific M2+ or M2- module in POR.

In order to investigate the organization of inputs and outputs of POR in detail, it was necessary to determine the boundaries of POR. Previous studies have used cytoarchitectonic and chemoarchitectonic boundaries, including staining for acetylcholinesterase and Timm’s stain, to define the boundaries of POR\textsuperscript{35}. However, these labels do not sharply delineate POR from all neighboring areas, and may not align with retinotopically defined boundaries, which are important features used for differentiating visual areas\textsuperscript{70–72}. To address this problem, Chapter 2 first describes multiple experiments which I performed to determine gene expression and connectivity patterns which label these borders.
1.3 LOCOMOTION AND VISUAL PROCESSING

A prominent behavioral state-based modulation of visual cortex activity is locomotion modulation. Locomotion modulation consists of an increase in the visual response gain and spontaneous firing rate of excitatory neurons during periods of running. This provides multiple benefits for visual processing. Increased spike counts during visual responses improve the fidelity of stimulus representation in V1, increasing the mutual information between stimulus identity and population activity. Locomotion improves the signal-to-noise ratio of visual responses by reducing baseline fluctuations of membrane potential. Stimulus discriminability is also improved by reducing noise correlations of neural responses within a cortical column. This mechanism bears resemblance to the decorrelation of neural responses which accompanies spatial attention.

Locomotion also reduces surround suppression in V1, possibly by changing the effective connectivity between pyramidal and somatostatin-positive interneurons. While this effect would seem to reduce discriminability of closely spaced stimuli, it may allow for integration of stimulus information across longer distances in the visual field. This supposition is supported by behavioral experiments showing that locomotion improves detection of full-field stimuli and visual targets presented at unpredictable locations in the visual field, but impairs detection of a small target presented at a fixed location.

Locomotion modulation plays a role in plasticity, as visual responses are restored more completely after visual deprivation if stimuli are paired with locomotion. After a period of dark
rearing, locomotion likewise accelerates the development of normal visual responses\textsuperscript{88}. This role in plasticity was demonstrated behaviorally, as locomotion improves performance recovery on a maze running task after visual cortex lesioning\textsuperscript{89}.

Locomotion modulation depends on long range inputs and inhibitory circuits within V1. Vasointestinal peptide-positive (VIP) inhibitory interneurons play a central role in locomotion modulation of pyramidal cells, as genetic deletion, pharmacological suppression, or photoablation of VIP cells eliminates most locomotion modulation in V1 excitatory neurons\textsuperscript{79,90,91}. Somatostatin-positive (SST) interneurons are also an important component in this circuit, although there appear to be multiple subtypes of SST cells with opposed physiological responses to locomotion. One SST type has suppressed activity during locomotion\textsuperscript{90,92,93}. This SST population inhibits pyramidal cells and is inhibited by VIP cells. Thus locomotion excites VIP cells through long range inputs, which suppresses SST cells, releasing inhibition on pyramidal cells, resulting in locomotion modulation of pyramidal cells. A second type of SST cell has increased activity during locomotion\textsuperscript{92–94}. This type forms a smaller proportion of the overall SST population\textsuperscript{93} and is less well understood as a driver of locomotion modulation. It is proposed to inhibit pyramidal cell-targeting parvalbumin-positive inhibitory interneurons, resulting in locomotion modulation of pyramidal cells through a double inhibitory circuit\textsuperscript{92,95}.

These mechanisms within V1 depend on external inputs signaling the presence or absence of locomotion. The mesencephalic locomotor region, a midbrain structure responsible for the initiation of locomotion, is one source of this signal\textsuperscript{96}. The LP, a higher visual thalamic nucleus, provides locomotion-modulated input to apical dendrites in L1\textsuperscript{17}. Cells within the dLGN were
originally thought to not be locomotion modulated\(^7\), however, subsequent research found cells within dLGN and dLGN→V1 axons which are locomotion modulated\(^17,78,97\). These dLGN inputs target L1 of V1 and likely originate from a subnucleus of the dLGN, the dLGN shell, which receives direct input from ON-OFF direction-selective retinal ganglion cells\(^{16,98,99}\).

Cortico-cortical feedback also provides locomotion-modulated inputs to V1. Secondary motor cortex (MOs), a hub for initiating and planning of motor output, provides locomotion modulated feedback to V1, which may serve as efference copy\(^{100}\). Anterior cingulate cortex, an important area for spatial learning and navigation\(^{101,102}\), provides similar inputs to V1, possibly to enhance plasticity for the formation of visuospatial memories\(^{100}\). In addition to these excitatory inputs, neuromodulation contributes to locomotion modulation, as demonstrated by reduced locomotion modulation following application of noradrenergic blockers\(^7\).

A striking feature of mouse V1 is that higher-order variables related to self-motion and navigation are represented in the activity of its cells. V1 activity in mouse represents these variables more explicitly than V1 of mammals with high acuity visual systems, such as primates. This is likely a result of the relatively limited number of levels in the mouse visual cortex hierarchy\(^34\), which results in V1 being hierarchically closer to cortical areas responsible for driving behavior\(^103\). Neural tuning for these properties in V1 cells have mostly been discovered by placing mice in a virtual reality environment, manipulating visual feedback based on locomotion velocity on a treadmill, and measuring for V1 activity modulations based on spatial variables or responses to optic flow\(^{104}\). In full virtual reality environments, V1 neurons have shown tuning for perceived virtual location\(^{105–107}\), expected objects that will soon be onscreen\(^{108}\),
and distance travelled\textsuperscript{106}. When simple optic flow stimuli are presented, neurons have been found that are tuned for optic flow speed\textsuperscript{109,110}, run speed\textsuperscript{110}, and visuomotor mismatch\textsuperscript{74,100,109,111}.

The relationship between locomotion modulation and V1 tuning for navigation-related variables is unclear, though they are necessarily intertwined, as they both involve changes in neural activity dependent on self-motion. It may be that locomotion modulation serves as an efference copy, to detect violations of expected optic flow\textsuperscript{88,112} or as a subtractive signal to eliminate the visual frame shift accompanying self-motion\textsuperscript{113}. The latter possibility is supported by the finding that V1 visual responses are suppressed during head movements\textsuperscript{114}. This role cannot completely explain locomotion modulation however, as many cells’ locomotion modulations do not linearly reflect run speed\textsuperscript{75}; for example, some cells show a transient increase in activity at or before locomotion onset\textsuperscript{79}. In Chapter 3, I do not make specific assumptions about the signal carried by V1 locomotion modulation. Instead, I treat locomotion modulation as putting a population of V1 cells into an advantageous physiological state for visual processing, via the mechanisms described above. This state may also include temporarily changing functional connectivity\textsuperscript{84} to favor detecting certain types of stimuli encountered during locomotion, such as wide field stimuli or optic flow.

The anatomy and behavioral state dependence of locomotion modulation demonstrate that it is a feedback-driven contextual adaption. As a widespread but heterogeneous attribute of V1 excitatory neurons, it presented a promising phenomenon through which to study the potential modular organization of top-down contextual modulation. Certain features of one of the M2 modules, M2- interpatches, suggested that locomotion modulation would be found to a greater
degree in these cells than in M2+ patches. M2- interpatch cells receive stronger inhibition than M2+ patch cells do from neighboring interneurons\textsuperscript{25}, suggesting that the VIP$\rightarrow$SST$\rightarrow$pyramidal and SST$\rightarrow$PV$\rightarrow$pyramidal circuits for locomotion modulation may have a greater modulatory effect on M2- interpatch cells. M2- interpatches showed specialized tuning for moving stimuli\textsuperscript{23}, suggesting a role in processing stimuli encountered during locomotion. Weaker orientation tuning, a property of M2- interpatches\textsuperscript{23}, was found in a physiologically defined class of V1 cells with high levels of locomotion modulation\textsuperscript{115}. M2+ patches and M2- interpatches also receive preferential inputs from dLGN and LP, respectively, which convey distinct signals in the presence of locomotion and visual stimulation\textsuperscript{17}.

In Chapter 3, I test for M2 module-specific responses to locomotion by performing calcium imaging of M2+ patches and M2- interpatches in mice freely locomoting while being presented with visual stimuli. After finding greater locomotion modulation and a distinct spatial distribution of noise correlations in M2- interpatches, I investigate anatomical features which might underlie these differences. I describe greater inputs from SST interneurons and MOs feedback projections to M2- interpatches, which may contribute to the observed physiological differences. Finally, I show that MOp-projecting cells and their apical dendrites align with M2-interpatches. This is the first demonstration of module-specific contextual modulation in visual cortex, and reveals an output stream from V1 that may be specialized for visual guidance of locomotion.
1.4 REFERENCES


Chapter 2: Modular Organization of Circuits for Affective Control of Navigation in Mouse Postrhinal Visual Cortex

2.1 ABSTRACT

The postrhinal area, POR, is a known center for integrating spatial with non-spatial visual information and a possible hub for influencing navigation by affective input from the amygdala. This may involve specific circuits within type 2 muscarinic acetylcholine receptor (M2)-positive or M2-negative modules of POR which associate inputs from the thalamus, cortex, and amygdala and send outputs to the entorhinal cortex. Using anterograde and retrograde labeling with conventional and viral tracers we found that all higher visual areas of the ventral cortical stream project to the amygdala, while such inputs are absent from primary visual cortex (V1) and dorsal stream areas. Unexpectedly for the presumed salt-and pepper organization of mouse extrastriate cortex, tracing results show that inputs from the dLGN (dorsal lateral geniculate nucleus) and LP (lateral posterior nucleus) were spatially clustered in layer (L) 1 and overlapped in M2+ patches of POR. In contrast, input from the amygdala to L1 of POR terminated in M2- interpatches. Importantly, the amygdalocortical input to M2- interpatches in L1 overlapped precisely with spatially clustered apical dendrites of POR neurons projecting to amygdala and medial entorhinal cortex (ENTm). The results suggest that circuits in POR, used to build spatial maps for navigation, do not receive direct thalamocortical inputs to M2+ patches. Instead they involve local networks of M2- interpatches which are influenced by affective information from the amygdala and project to ENTm, which drives navigation.
2.2 SIGNIFICANCE

A central purpose of visual object recognition is identifying the salience of objects and approaching or avoiding them. However, it is not currently known how the visual cortex integrates the multiple streams of information, including affective and navigational cues, which are required to accomplish this task. We find that in a higher visual area, the postrhinal cortex, the cortical sheet is divided into interdigitating modules receiving distinct inputs from visual and emotion-related sources. One of these modules is preferentially connected with the amygdala and provides outputs to entorhinal cortex, constituting a processing stream that may assign emotional salience to objects and landmarks for the guidance of goal-directed navigation.

2.3 INTRODUCTION

The chief concern for survival is to detect danger and react to it\(^1\). In rodents, defensive strategies include the reflexive startle response, freezing when danger is distant and escaping when it is close. To do this effectively it is important to know the spatial layout of the local and distal environment. For this, animals rely on neural circuits connecting multiple brain structures including the amygdala, which drives flight, freezing, and approach\(^2,3\), and areas of the limbic, prefrontal and sensory cortex\(^4\). In rodents a critical structure is the postrhinal area, POR\(^5,6\), which is part of the homologous primate parahippocampal cortex\(^7\) and is a higher center for visual scene processing\(^8,9\). Studies in rats have shown that POR integrates visual inputs to extract contextual information\(^10\). It also transforms egocentric into allocentric head direction information, which is thought to form the basis for the grid-cell metric in the downstream ENTh\(^10-13\). The network for spatial processing is often contrasted with a pathway that carries preferentially, but not exclusively, non-spatial information to the hippocampus through lateral
The entorhinal cortex (ENTI) via the perirhinal (PERI) multimodal association cortex\textsuperscript{14–17}. The second major function of POR is the association of visual objects with reward and context-dependent fear memory via a circuit formed with the amygdala\textsuperscript{18,19}. This pathway is known as the ‘cortical route’ for visual fear response, which has been contrasted with the shorter latency ‘subcortical route’ for aversive responses\textsuperscript{18,20–22}. A major source of the subcortical input channel is the superior colliculus (SC) which connects through the lateral posterior nucleus (i.e. pulvinar) to the lateral amygdala (LA), where it triggers defensive responses to visual threats, such as looming stimuli\textsuperscript{23}. This information may be used in the reciprocal circuit with POR\textsuperscript{19} to associate affective significance to objects and places\textsuperscript{10}. A second branch of the pulvinocortical pathway sends input directly to POR and provides for its high sensitivity to fast moving dot stimuli\textsuperscript{12,24}. Optical recordings of calcium transients in V1 have shown that LP terminals in L1 carry signals for unexpected visual motion which are incongruent with the animal’s running speed\textsuperscript{25}. These responses suggest that locomotion-related cues may be integrated in POR, enabling the detection of moving objects during self-motion. Direct input to POR also comes from the dLGN\textsuperscript{26}, whose terminals in L1 of V1 are most active when the running speed matches the optic flow\textsuperscript{25}, providing distance-tracking cues for path integration useful for determining the position relative to landmarks.

We have shown previously in flatmounted mouse POR that M2 expression in L1 is patchy\textsuperscript{27}. Single unit recordings have further shown that L2/3 neurons aligned with M2+ patches are more sensitive to object features, while cells associated with M2- interpatches are more sharply tuned to stimuli moving at high speed\textsuperscript{27}. D’Souza et al., (2019)\textsuperscript{28} have demonstrated that this pattern is reflected in the organization of dLGN inputs to M2+ patches and LP projections to M2-
interpatches of V1. These findings raised the question whether the modular organization of thalamocortical inputs are preserved in POR and whether inputs from the amygdala overlap with output neurons projecting to the amygdala and ENTm. To address these questions we have used conventional and viral anterograde and retrograde tracers of connections to and from POR. The results show that POR receives mainly inputs from areas of the ventral cortical processing stream$^{29}$. dLGN and LP projections to L1 overlap in M2+ patches whereas inputs from the amygdala terminate in M2- interpatches. The spatial overlap of amygdalocortical inputs with the apical dendritic tufts of POR neurons projecting to the amygdala and ENTm suggests that M2-modules provide a substrate for the affective modulation of POR output used for navigation.

2.4 MATERIALS AND METHODS

Animals

Experiments were performed in 5-10 week-old male and female C57BL/6J, Ai9 (Gt[ROSA]26Sortm9(CAG-tdTomato)Hze)/J, PV-Cre (Bg.129P2-PvalbTm(CRE)Arbr)/J, x Ai9 (Gt[ROSA]26Sortm9(CAG-tdTomato)Hze)/J and Chrm2-tdT-D knock-in (BG6.Cg-Chrm2tm1.1Hzc)/J mice. All experimental procedures were performed in accordance with the National Institutes of Health guidelines and under the approval of the Washington University Institutional Animal Care and Use Committee.

Surgical procedures

Mice were anesthetized with a Ketamine/Xylazine mixture (86/13 mg/kg, IP). Analgesia was achieved by presurgical injections of Buprenorphine-SR (0.1 mg/kg, SubQ). Ocular ointment was applied to protect the cornea. Mice were head-fixed in a stereotaxic frame. Body
temperature was maintained at 37°C. For each injection site, a small craniotomy was made over the target. Tracer injections were performed through glass micropipettes (15-20µm tip diameter) and, depending on the tracer used, attached to either a Picospritzer (for Bisbenzimide and Diamidino Yellow [DY]), a Iontophoresis current source (for Biotinylated Dextran Amine [BDA]), or a Nanoject II pump (for adeno associated viruses [AAVs]). At each cortical site, two injections were made, one 0.5 the other 0.3 mm below the pial surface. After injections were complete, pipettes remained in place for 3 min before they were retracted. The scalp was stapled closed with wound clips.

**Combined anterograde tracing with BDA and retrograde tracing with Bisbenzimide**

For mapping axonal projections from visual cortex to the amygdala in C57BL/6J mice, BDA (10,000 molecular weight, 5% in H₂O; Invitrogen) was injected through a glass micropipette using iontophoresis (Midgard/Stoelting, 7s duty cycle for 7 min, 3µA). Injections were made into the left hemisphere (in mm anterior to transverse sinus/lateral to midsagittal suture): V1 (1.1/2.8), LM (1.4/4.0), P (1.0/4.2), L1 (1.45/4.2), POR (1.15/4.3), AL (2.4/3.7), RL (2.8/3.3), PM (1.9/1.6), AM (3.0/1.7), A (3.4/2.4). In the same animal, callosal connections were retrogradely labeled by blanketing the right visual cortex with 20-30 injections (20 nl each) of Bisbenzimide (5% in H₂O, Sigma). After 3 days of survival, mice were euthanized with an overdose of Ketamine/Xylazine (500/50mg/kg, IP) and perfused with 4% paraformaldehyde (PFA, 0.1M phosphate buffer, pH7.4 [PB]). The brain was extracted from the cranium and cryoprotected in 30% sucrose (PB). The Bisbenzimide-labeled callosal projection pattern was imaged *in situ* with a stereomicroscope (Leica MZ16F) equipped with fluorescence optics (excitation 338 nm, emission 505 nm). The injection site was visible as a weakly fluorescent
spot, whose location relative to the callosal pattern was used for areal identification. Coronal sections (50 μm) were cut on a cryostat and stained for BDA using an ABC reaction (Vectastain ABC Elite). Sections containing the brainstem, diencephalon, and the amygdala were mounted on glass slides, and after dehydration in Ethanol and clearing in Xylenes, the Diaminobenzidine reaction product was intensified with AgNO₃ and HAuCl. Representative sections were stained with Cresyl Violet for Nissl substance. The slides were coverslipped with mounting medium (DPX, Sigma) and imaged under a microscope equipped with darkfield optics. Images were captured with a CCD camera (CoolSnap EZ, Roper Scientific). For analyses of the termination pattern in each nucleus, coronal sections were imaged under 10X magnification, and grayscale images recorded with MetaMorph NX2.0 acquisition software (Molecular Devices). Optical density of BDA labeled axons were generated using custom written Matlab scripts. A circular averaging 2D filter was used to blur the raw image, and contours denoting distinct optical density levels were generated. Optical densities were calculated using ImageJ. The optical density in a specific nucleus was computed as a percentage of total input to all subcortical projection targets. The relative optical density was found by computing the density relative to the maximal density at the injection site. The two-tailed t-test was used for statistical comparison.

**Retrograde tracing with Diamidino Yellow**

To determine the sources of cortical and subcortical inputs to POR, neurons were retrogradely labeled with the fluorescent tracer DY (2% in H₂O, 50nl; EMS-Chemie). Injections into POR (in mm anterior to transverse sinus/lateral to midsagittal suture, 1.15/4.3) were made in PVtdT mice whose pattern of tdT fluorescence was used to identify cortical areas and subcortical nuclei. After 3 days of survival, mice were perfused with 1% PFA. Brains were extracted, then the
cortex including the hippocampus were detached from the rest of the brain and flatmounted\textsuperscript{27}. The tissue was postfixed in 4\% PFA (overnight, 4\(^\circ\)C), followed by overnight immersion in 30\% sucrose (PB, 4\(^\circ\)C). Tangential sections were cut at 40 \(\mu\)m on a freezing microtome, wet mounted on glass slides, and coverslipped in PB. Images were captured with a CCD camera (Lumenera Infinity\textsuperscript{S3}-URM, Teledyne) and Metamorph NX2.0 acquisition software (Molecular Devices) using a stereomicroscope (Leica MZ16F) or an epifluorescence microscope (Nikon 80i), respectively, equipped with UV fluorescence (excitation 355-425 nm, emission 470 nm) and tdT (excitation 520-600 nm, emission 570-720 nm) optics. For storage, sections were mounted on glass slides and coverslipped with DPX.

\textit{Anterograde tracing with AAVs}

For retinotopic mapping of POR, side-by-side injections with AAV.2/1.hSynapsin.EGFP.WPRE.bGH (Penn Vector Core, 46 nl) and AAV.2/1.hSynapsin.tdTomato.WPRE (Penn Vector Core, 46 nl) were made at select combinations of retinotopic locations in V1 of C57BL/6J mice. The injections were confined to a space within (in mm anterior to transverse sinus/lateral to midsagittal suture) 1.0-1.6/2.7-1.4/3.3. The same viral tracers and injection volumes were used to trace inputs from the subcortical structures (in mm posterior of bregma/lateral of midline/below pial surface): lateral amygdala (LA, 1.8/3.55/3.7), dLGN (2.35/2.15/2.55) and LP (1.85/1.25/2.65) in C57BL/6J or Chrm2-tdT mice. After 14-18 days of survival mice were euthanized and perfused with 1\% PFA. The cortex of V1-, dLGN- and LP-injected mice was flatmounted, postfixed in 4\% PFA, cryoprotected in 30\% sucrose and stored overnight with the rest of the brain. In amygdala-injected mice, neocortex was separated from the limbic structures along the rhinal fissure, flatmounted,
postfixed, cryoprotected and sectioned at 40 um in the tangential plane on a freezing microtome. The amygdala was sliced in the standard coronal plane. For visualization of M2 expression in C57BL/6J mice, tangential and coronal sections through cortex were immunostained with an antibody against M2. This procedure involved preincubation in blocking solution (0.1% TritonX-100, 10% normal goat serum, PB), followed by incubation in primary rat anti-M2 antibody (1:500, MAB367 [Millipore], 48h at 4ºC) and reaction with Alexa-647-labeled goat anti-rat secondary antibody (1:500, A21247, Invitrogen). M2 labeling patterns in Chrm2-tdT mice were identified by tdT fluorescence. Selected sections through cortex and amygdala were counterstained with NeuroTrace 435/455 (Molecular Probes). Anterogradely labeled axons and M2 patches were imaged at 4X and 10X under an epifluorescence microscope equipped with EGFP, tdT, and IR (excitation 650, emission 665) fluorescence optics, a CCD camera and acquisition software. In cortical sections, POR and PORa were outlined based on M2 expression. Above-background EGFP or tdT fluorescence intensity (per pixel) in POR and PORa was determined in a series of sections. Normalized intensity for each case was computed by dividing background-subtracted intensity by the maximum background-subtracted intensity density found in this case.

To disambiguate laminar identification based on cytoarchitecture (i.e. counterstaining with NeuroTrace 435/455) in flatmounted cortex, we immunostained tangential sections of flatmounted cortex of C57BL/6J mice for the transcription factor CTIP2 (COUP TF-interaction protein) which selectively labels L5 and L6<sup>32</sup>. Immunostaining was performed with a rat anti-CTIP2 antibody (1:500, Abcam 18465) which was visualized with an Alexa-488-labeled secondary goat anti-rat IgG (1:500, Invitrogen).
**Retrograde tracing with AAVs**

To investigate the tangential distribution of POR neurons that project to the amygdala and the entorhinal cortex we used retrograde tracing with rAAV2-Retro/CAG-Cre (University of North Carolina, Vector Core) in Ai9 mice. Injections (46 nl) were made into the lateral amygdala (LA, BLA) (in mm posterior of bregma/lateral of midline/below pial surface) 1.8/3.55/3.7, and ENTm (in mm anterior to transverse sinus/lateral to midline/below the pial surface) 0.7/5.25/3.55 angled 40º from vertical. After 14-18 days of survival, perfusion with 1% PFA, flatmounting of cortex, postfixation in 4% PFA, cryoprotection and cutting in the tangential plane, sections were immunostained with rat anti-M2 and Alexa-647-labeled goat anti-rat antibodies. Sections were wet mounted on glass slides and imaged under IR and tdT illumination at 4X-40X magnification to identify M2 patches, cell bodies, dendrites, spines and local axons of retrogradely labeled cells in POR.

**Analysis of patchy M2 expression**

M2+ patches and M2- interpatches were delineated from images of immuolabeled (in C57BL/6J mice) or the expression of Chrm2-tdT (in Chrm2-tdT mice) using custom Matlab scripts. M2 expression images were spatially normalized by dividing the intensity of every pixel by the mean intensity within a 100 µm radius, images were blurred with the Matlab ‘nanconv’ function (Figure 2.4D). Images were then divided into six quantiles based on pixel intensity (Figure 2.4E). These borders were overlaid onto images of virally anterogradely labeled axons or retrogradely labeled cell bodies and dendrites. Comparison of M2 immunostaining and Chmr2-tdT expression demonstrated that the two methods produced nearly identical borders of M2+ patches and areal boundaries, thus the same analyses were used for both. This similarity was verified by
performing M2 immunostaining in Chrm2-tdT mice and overlaying M2 expression patterns from both markers in the same section.

Analysis of projection strength

For the quantification of M2+ patch:M2- interpatch intensity ratios of anterogradely labeled axonal projections, M2 expression images were first high pass filtered, blurred, and divided into 6 intensity quantiles of equal area (Figures 2.4D, E). The lower 3 quantiles were treated as M2-interpatches and the upper 3 quantiles treated as M2+ patches. The mean normalized EGFP or tdT fluorescence intensity within patches and interpatches was found for each case. Multiple cases were then averaged together to obtain a mean and standard error for the M2 patch:interpatch intensity ratio in each pathway (Figure 2.5E).

Analysis of dendrite distribution

Images of M2 immunofluorescence or Chmr2-tdT expression were acquired and divided into six quantiles based on M2 expression intensity. Apical dendrites in L1/2 of retrogradely labeled pyramidal cells in POR were identified by size (0.4-1.5 µm in diameter) and their tapered, spiny morphology. The dendrites were traced manually at 40X magnification and their total length within each quantile of M2 expression was computed. For each quantile in each mouse, the proportion of dendrite length out of all dendrites within that image was computed, and the Pearson correlation coefficient between quantile level and proportion of dendrites was computed. Images from 2 mice were analyzed for POR→ENTm projections and 3 mice were analyzed for POR→LA projections.
**Analysis of laminar distribution of retrogradely labeled cells**

Series of flat-mounted tangential sections were taken from mice in which rAAV2-Retro/CAG-Cre had been injected into either ENTm (N = 2 mice) or LA (N = 2 mice) (Figure 2.7I, J). Epifluorescence images of M2 expression and retrogradely tdT-labeled cells were taken at 4X and overlaid, centered on the posterior cortical pole. In each image, borders for LM, LI, P, POR, and PORa were manually drawn based on M2 immunolabeling visualized with Alexa 647 IgG and all tdT-labeled cells were marked. Custom Matlab scripts were used to tabulate counts of cells within each area. Cell counts for unusable sections were interpolated using Akima piecewise cubic interpolation with the Matlab ‘interp1’ function. To compensate for variations in labeling effectiveness across cases, cell counts were normalized for each mouse by dividing by the maximum number of cells in any one area in any one section of that mouse.

### 2.5 RESULTS

*POR is contained within a M2+ region of parahippocampal cortex*

The borders of POR have been outlined previously by mapping of retinotopic inputs from V1. However, it remained unknown whether its borders align with the expression of M2, an effective marker used to identify cortical areas. Additionally, the original description of POR by Burwell’s group was derived from cytoarchitectonic features observed in coronal sections. This raised the question whether the borders seen in coronal sections and flatmounted cortex are aligned with one another. To find out, we combined multicolor viral tracing of retinotopic projections from V1 with the expression patterns of M2 and parvalbumin-tdTomato (PVtdT). We then analyzed the axonal projections in tangential sections through the flatmounted cortical hemisphere. Reconstructions of the retinotopic map were done from serial sections through the...
cortical mantle, eliminating the challenging task of aligning sequential coronal sections. In tangential sections through L4 stained with an antibody against M2, we identified V1 as an intensely immunopositive triangular area surrounded by the more weakly fluorescent extrastriate cortex (Figure 2.1A, inset). Lateral to V1, M2 stained a boot-shaped region which overlapped with the ventral stream areas LM (lateromedial), LI (laterointermediate), POR, and PORa (POR anterior)\(^\text{31}\). M2 expression dropped sharply at a M2- strip along the rhinal fissure, which contained ECT (ectorhinal area) and P (posterior area) and was continuous with TEp (temporal area, posterior) on the anterior-dorsal side of POR and PORa. A similar boot-shaped pattern was seen in the expression of PVtdT in PVcre x Ai9 mice (Figure 2.1D) which overlapped with M2 (data not shown)\(^\text{31}\).

To outline the borders of areas within the M2+ boot we simultaneously injected C57BL/6J mice (N = 5) with two anterograde viral tracers, AAV.2/1.hSynapsin.EGFP (AAV.EGFP) and AAV.2/1.hSynapsin.tdTomato (AAV.tdT) at different visuotopic locations of V1. The results of one such case in which AAV.EGFP was injected near the intersection of the vertical and horizontal meridian\(^\text{34}\) and AAV.tdT was targeted to the upper temporal visual field\(^\text{30}\) are illustrated in Figures 2.1A-C. The results show that axonal projections terminated in multiple pairs of red and green patches distributed across the surrounding extrastriate cortex (Figures 2.1B, C). Anterior-dorsal to the rhinal fissure, we found three pairs of patches: two within the M2+ boot and one in the M2- strip. Reading the map from posterior to anterior starting at the border of V1, the colors of the projections switch from red to green, reverse back to red, and then reverse again to label a fainter patch of more interspersed red and green fibers. Because map reversals have been interpreted as areal borders\(^\text{34,35}\), we concluded that the
Figure 2.1 Mapping areas of mouse parahippocampal cortex. A, Tangential section through flatmounted postrhinal cortex stained with an antibody against M2 muscarinic acetylcholine receptor (magenta). Inset shows low magnification image of M2 expression in L4 of V1 and surrounding cortex. Green and red spots indicate AAV2/1.hSyn.EGFP (green) and AAV2/1.hSyn.tdT (red), injection sites into nasal/central and peripheral/upper visual field representations, respectively. B, C, Anterogradely labeled clusters of axon terminals labeled after AAV injections into V1 (A, inset) show retinotopic maps in LM, LI, P, POR, and PORa. The overlay reveals that projection maps in POR and PORa are contained within the M2+ cortex (C). D, Expression of PVtdT in postrhinal cortex shows a foot-shaped pattern similar to M2 expression in C. M2 and PVtdT expression is extremely sparse in P, ECT and TEa. E, F, Coronal section from Chmr2-tdT mouse (approximate location indicated by green line in (A) shows sharp transitions of laminar M2 pattern at the POR/ECT and ECT/ENTm borders (E). The transitions coincide with a loss of L4 in ECT shown by counterstaining with NeuroTrace 435/455 (F).
red-to-green/green-to-red and the green-to-red/red-to-green transitions mark the P/POR and the POR/PORa borders, respectively\textsuperscript{30,36}. Notably, injections at higher retinotopic altitudes shifted the labeled patches in both P and POR closer to the borders with LM and LI, while injections below the horizontal meridian failed to push POR projections out of the M2+ boot into the M2- strip. This pattern indicates that the POR/ECT border coincides with the posterior margin of the M2+ boot, which agrees with the limited lower field representation in POR\textsuperscript{34}.

How do the borders of POR observed in the tangential plane compare to those reported by Beaudin et al., (2013) in coronal sections? To find out we used Chrm2-tdT mice and counterstained coronal sections with NeuroTrace 435/455, a blue fluorescent Nissl stain of ribosomal RNA. Figure 2.1E shows a section from an approximate location indicated by the green line on the flatmap depicted in Figure 2.1A. It is evident that the multilayered M2 expression in the dorsal part is interrupted suddenly by an unstained gap and then reappears as a simpler pattern in more ventral parts of the section. In tangential sections the unstained gap coincides precisely with the M2- band along the rhinal fissure. A more direct comparison with previous cytoarchitectonically defined borders shows that the gap also overlaps with a region in which L4 is no longer visible (Figure 2.1F). This gap was previously identified as ventral POR (PORv)\textsuperscript{6}. Our analysis shows that the gap coincides with the M2- band found in tangential sections and suggests that PORv\textsuperscript{6} is not part of POR but falls into ECT which borders M2+ POR cortex.
M2+ patches in higher visual areas are linked to cortical magnification

We have shown previously that alternating M2+ and M2- patches are not unique to V1, but also exist in L1 of LM, LI, POR, PORa, auditory (AUD), and retrosplenial (RSP) cortex27. We in addition noted that in each of these areas M2 expression is more uniform in layers below the L1/2 border (Figures 2.2A, B). In V1 M2+ patches parcellate the plexiform L1 in the tangential plane into constant-size domains, which extend to the cellular L2/3, and in groups of 4-8 represent the visual point image27. This organization raised the question of whether the size, shape, and density of M2+ patches is related to the layout of the visuotopic maps in LM, LI, and POR. We determined the aspect ratio of M2+ patches along the major and minor axes of V1, LM, LI, and POR measured from Figure 2.7 of Garrett et al., (2014). We found that in all four areas, average patch anisotropy was aligned with the axes of the containing area (Figure 2.2D). In areas V1, LI, and LM, whose major axes represent altitude, patches were stretched along elevation. In POR, with the major axis mapping azimuth, average patch elongation occurred along latitude. In V1, LM, and LI, patch dimensions closely matched those of their containing area, with major:minor length for patches (V1 1.21 ± 0.03 [12 mice, 1354 patches], LM 1:1.4 ± 0.05 [7 mice, 122 patches], LI 1:1.42 ± 0.08 [7 mice, 78 patches], POR 1.21 ± 0.03 [16 mice, 278 patches]) and areas (V1 1.22, LM 1:1.53, LI 1:1.53, POR 1: 1.59)34. These results suggest that anisotropies in the representation of retinotopic space are shared by the M2+ patches within the areas we have examined.

Next, we determined the spatial distribution of M2+ patches and found that the density was similar in V1, LM, and LI and only slightly lower (p < 0.05, t-test) in POR (Figure 2.2E). These results indicate that on average the patch size (top 20% of M2+ pixels) is relatively constant
Figure 2.2 Patchy M2 expression in higher cortical areas. A, B, Tangential section through occipital cortex of Chrm2-tdT mouse shows patchy M2 expression in L1 (A) and uniform expression in L4 (B) of V1, LM, LI, POR, PORa, AUD, and RSP. C, Higher magnification image of LM, LI, POR, and PORa showing that M2+ patches are elongated parallel to the major axes (yellow double headed arrows) of LM, LI, and POR. D, Mean ± SEM ratio of major:minor axial lengths of M2+ patches in V1, LM, LI, and POR (mean 63.2 patches/mouse analyzed, t-test). E, Mean ± SEM spatial density of M2+ patches across areas (same mice and patches as in D, t-test). F, Mean ± SEM M2+ patches per deg² of visual space, determined by multiplying spatial densities with magnification factor of respective area XXX (same mice and patches as in D, t-test).
across areas, as was the case across different quadrants of V1\textsuperscript{27}. How much of the visual field is represented by a M2+ patch in a given area? To find out we multiplied patch density (patches/mm\textsuperscript{2}) by the cortical magnification factor (mm\textsuperscript{2}/deg\textsuperscript{2})\textsuperscript{34} and obtained a quantity of patches/deg\textsuperscript{2} in V1, LM, LI, and POR. We found that a 10x10 degree-wide field of visual space, which accounts for about the size of the point image in V1 and LM\textsuperscript{9}, contains 3.93 ± 0.08 (12 mice) and 3.46 ± 0.23 (7 mice) patches/mm\textsuperscript{2}, respectively. In LI and POR, a similar 10x10 degree-wide field contained about half the number of patches (LI 7 mice, POR 16 mice), indicating that a single LI or POR patch represents a proportionately larger amount of visual space than in V1 and LM (Figure 2.2F).

\textit{Anterogradely labeled projections from visual cortex to amygdala}

Despite massive efforts\textsuperscript{37–39}, there are no detailed descriptions of the connectivity of visual cortical areas with the amygdala in mice. In all previous studies, injections involved multiple areas and areal assignments were based on the computed majority stake, which is known to underestimate the connectome density\textsuperscript{31}. We have taken a different approach in which we only accepted clean hits of the source area for anterograde tracing with BDA. To do this we relied on the complementary patterns of M2 expression and callosal connections\textsuperscript{36}. Callosal connections were labeled by retrograde transport of Bisbenzimide, and visualized \textit{in situ} in fixed brains imaged under a fluorescence stereomicroscope. This procedure also revealed the injection site as a bright spot which was, based on the distinctive callosal projection pattern, readily assigned to V1, PM, AM, A, RL, AL, LM, LI, P, or POR (Wang and Burkhalter, 2007; Figures 2.3A-J). The
Figure 2.3 Axonal projections of visual cortex→amygdala pathways. A-J, In situ images of callosal connections traced by retrograde transport of Bisbenzimide from the posterior third of the right hemisphere. The arrows point to bright spots which represent the BDA injection sites in V1, LM, P, LI, POR, AL, RL, PM, AM, and A of the left hemisphere. A'-J’, Darkfield images of coronal sections showing anterogradely BDA labeled axonal projections (yellow) from V1, ventral (LM, P, LI, POR) and dorsal (AL, RL, PM, AM, A) stream areas terminating in different nuclei of the amygdala. Note that the white structures represent unlabeled, highly reflective myelinated fibers of the internal capsule (int). Scales: A-J (1mm), A'-J' (0.5 mm).
cortex dorsal to the rhinal fissure was then separated from the brain, flattened, cut tangentially, reacted for M2 immunofluorescence and stained with an ABC reaction to reveal the BDA injection site. Injections were considered clean hits if they were confined within the areal boundaries delineated by Gămănuţ et al., (2018). The rest of the brain was cut coronally and sections containing the amygdala were stained to reveal BDA labeled axonal projections. We found that the projections from V1 and the dorsal stream areas, AL, RL, PM, AM, and A, were extremely weak and too sparse for determining their optical density (Figures 2.3F’-J’). In sharp contrast the projections from the ventral stream areas, LM, P, LI, and POR, were much denser (Figures 2.3A’-E’). The vast majority (98-100%) of inputs from LM, LI, and P to the amygdala terminated in LA with only minor offshoots to BLA (basolateral amygdala) (Figures 2.S1B-E inset). Projections from POR were percentage-wise (percent of total input to amygdala and percent optical density relative to injection site) strongest in LA but, compared to LM, LI, and P, a larger fraction terminated in BLA and CeA (central amygdala).

Compared to the total output from 10 visual cortical areas to 31 subcortical targets, the weight of amygdala projections was weak and even the strongest combined input from POR to LA and BLA was only ~10% of the total strength (Figure 2.S1E). Notably, POR input to another emotion regulatory center for fear, the dorsal lateral periaqueductal grey (DLPAG), which responds to cortical stressors and unlike the amygdala does not receive visceral input was only about 2% (Figure 2.S1E). Similarly sparse projections to DLPAG were found from P, AM, and PM (Figures 2.S1D, H, I).

Bennett et al., (2019) have shown that the posterior part of LP is reciprocally interconnected with
Figure 2.S1 Relative strength of anterogradely BDA-labeled projections of mouse visual cortex to 31 subcortical targets. **A**-**E**, Average ± SEM optical density of projections from V1 and the ventral stream areas LM, LI, P, and POR in percent of the summed density of all subcortical projections labeled from each area. **F-J**, Projections from the dorsal stream areas AL, RL, AM, PM and A. Colors indicate functionally broadly similar groups of subcortical targets. **B-E insets**, Relative strength of anterogradely BDA labeled projections from ventral stream areas LM, LI, P, and POR to LA, BLA, BMA, CeL, and CeM of the amygdala. Percent (mean ± SEM) of total optical density of labeled terminal axonal branches (black bars). Background-subtracted percent (mean ± SEM) of optical density relative to the injection site (grey bars).
POR. We found a similar preference of POR for caudal LP, including a much weaker projection to dLGN (Figures 2.S1E). We in addition found retrogradely DY (Diamidino Yellow) labeled POR-projecting neurons in LP and the dLGN shell (Figures 2.6D, E), suggesting that intracortical communication through transthalamic circuits may not only go through the higher order LP but also involve the first order dLGN. The strong preference of the posterior parts of LP (LPLC, LPMC) was also seen in the inputs from the ventral stream areas LM, LI, and P (Figures 2.S1B-D). These projections were in striking contrast to the complete absence of inputs to the LPLC and LPMC from areas of the dorsal stream, AL, RL, AM, PM, and A (Figures 2.S1F-J). Inputs to the middle and anterior part of LP (LPLR, LPMR), including the laterodorsal thalamic nucleus (LDDM, LDVL), originated from ventral and dorsal stream areas (Figures 2.S1B-A). LD projects widely throughout areas of the dorsal and ventral visual processing streams. Weak POR projections were also found to the auditory MGV (ventral medial geniculate) and MZMG (marginal zone of medial geniculate) including the olfaction-related MDL (mediolateral thalamic nucleus), suggesting a role of these inputs in polymodal sensory processing (Figure 2.S1E). Unlike ventral stream areas, which more strongly project to oculomotor and motor coordination centers than ventral stream areas, such inputs were extremely weak or absent from POR (Figure 2.S1E). A notable exception were the strong POR inputs to the dorsomedial CP (caudoputamen) (Figure 2.S1E), which plays a role in goal-directed behavior.

Anterogradely labeled projections from amygdala terminate in M2- interpatches of POR

It has been known from studies in rat that projections from LA and BLA to POR terminate in L1-3 with weaker input to the layers below. To find out whether these inputs were modularly organized we traced inputs from LA and BLA (Figure 2.4B, inset) with AAV.EGFP or AAV.tdT
**Figure 2.4** Axonal projections of amygdala→POR pathway. A-C, Tangential section through L1 of flatmounted POR in Chrm2-tdT mouse showing non-uniform pattern of M2+ patches (magenta) and M2- interpatches (dark) (A). Patchy axonal projections to L1 labeled by anterograde tracing with AAV2/1.hSyn.EGFP from the lateral amygdala (LA) (B). Coronal section of injection site in LA (B inset). Overlay of A and B, shows that LA→POR projection to L1 terminates preferentially in M2- interpatches (C). D, Heat map of M2 expression in POR (A). E, Partitioning of heat map (D) into six equal-area quantiles (shades of gray), for delineation of M2+ patches and M2- interpatches. F, Normalized average ± SEM (N = 5 mice) EGFP fluorescence intensity in each of the 6 quantiles shows that LA→POR inputs are preferentially associated with M2- interpatches ($R = -0.86$, $p < 10^{-8}$, Pearson correlation).
in C57BL/6 or Chr2-tdTmice. We found that the laminar distribution of inputs from both sources were similar, which let us pool (LA/BLA, N = 2) the baseline-subtracted fluorescence intensity of axonal projections. Inputs were densest in L1, weaker in L3/4 and sparse in layers 2, 5 and 6 (Figure 2.5F). To study whether inputs specifically targeted M2+ patches or M2-interpatches, we removed the cortex dorsal to the rhinal fissure, flatmounted the tissue, cut tangential sections and sliced the amygdala in the standard coronal plane. Projections to L1 of POR were strikingly non-uniform, targeted M2-interpatches and largely avoided M2+ patches (Figures 2.4A-D). We performed an automated delineation of M2+ patch borders with custom Matlab scripts, using a procedure similar to that used by Sincich and Horton (2005)\textsuperscript{46}. M2 intensity images were high-pass filtered and blurred, then partitioned into six quantiles based on fluorescence intensity (Figures 2.4D, E). To test for different input strengths to patches and interpatches, EGFP intensity was compared across M2-defined quantiles in 5 mice (Figure 2.4F). EGFP intensity showed a strong negative correlation with M2 expression (R = -0.86, p < 10\textsuperscript{-8}, N = 5, Pearson correlation) and projection strength in the highest quantile (6) was 17% of that in the lowest quantile (1), indicating that M2-interpatches received significantly stronger input from the LA/BLA than M2+ patches.

**Anterogradely labeled projections from dLGN and LP terminate in M2+ patches of POR**

We have found recently that M2 expression in V1 divides L1 into interdigitating modules receiving dLGN input to M2+ patches and LP input to M2-interpatches\textsuperscript{27,28}. Both of these thalamic nuclei are also known to project to extrastriate visual cortex\textsuperscript{26,37} but only LP has been shown unequivocally to project to POR\textsuperscript{12,42,47,48}. Here, we investigated whether dLGN and LP project to POR and whether inputs to L1 show an interdigitating pattern similar to the one we
Figure 2.5 Axonal projections of dLGN→POR and LP→POR pathways. A, B, Immunostained M2+ patches in L1 of flatmounted tangential section through POR and PORa (A). dLGN→POR axonal projections traced anterogradely with AAV2/1.hSyn.EGFP (B). Coronal section of injection site in dLGN (B inset). White contours indicate borders between 4th and 5th intensity quantiles (out of 6) of M2 expression. Overlay of contours shows overlapping patterns of M2+ patches and dLGN→POR projections (B). C, D, AAV2/1.hSyn.EGFP labeled LP→POR projections (D) to M2+ patches (C) in L1 of POR. Coronal section of injection site in LP (D inset). E, Mean ± SEM patch:interpatch EGFP intensity ratio of dLGN→POR, LP→POR, LP→LM, dLGN→LM, dLGN→LI, LP→LI, LA/BLA→POR, and V1→POR axonal labeling in L1 of the projection target. F, Laminar distribution of anterogradely labeled LA/BLA→POR, dLGN→POR, and LP→POR projections.
have found in V1\textsuperscript{28}. For this purpose we injected the anterograde viral tracers (AAV.EGFP, AAV.tdT) either into LP or dLGN (Figures 2.5B, D inset). We found that input from dLGN and LP terminated in POR and PORa (Figures 2.5A-D; dLGN [N = 5], LP [N = 2]). dLGN input was densest in L1 and L5, moderate in L4 and L6 and weakest in L2/3 (Figure 2.5F). LP input was dense in L1 and L4, moderate in L5 and L6, and weakest in L2/3 (Figure 2.5F). To determine the patch:interpatch ratio of input to L1, the top three M2 quantiles were taken as M2+ patches and the bottom three quantiles as M2- interpatches. The results show that the tangential distribution of dLGN and LP inputs to L1 of POR and PORa (data not shown) overlapped with M2+ patches (patch:interpatch ratios dLGN→POR 3.04 ± 0.67; LP→POR 3.51 ± 0.42) (Figure 2.5E). The targeting in POR and PORa to M2+ patches distinguished dLGN and LP input from amygdala inputs, which terminated in M2- interpatches (Figures 2.4C, F). dLGN and LP inputs to LM and LI likewise targeted M2+ patches and avoided M2- interpatches (Figure 2.5E; patch:interpatch ratios dLGN→LM 2.45 ± 0.59 [N = 4]; LP→LM 2.11 ± 0.71 [N = 3]; dLGN→LI 2.09 ± 0.49 [N = 4]; LP→LI 2.1 ± 0.75 [N = 3]). In agreement with previous studies\textsuperscript{28}, inputs to V1 were preferentially targeted to M2+ patches, whereas LP inputs preferred M2- interpatches (data not shown). Inputs from V1 to POR, while topographical (Figures 2.1B, C), showed no clear preference for either compartment (patch:interpatch ratio 1:1 ± 0.03, [N = 4], p = 0.88, one-sample t-test) (Figure 2.5E).

Sources of neurons projecting to POR

To map the sources of inputs to POR, we retrogradely traced neurons in PVtdT and Chrm2-tdT mice with DY. Unsurprisingly, given the results from anterograde tracing (Figures 2.4A-C, 2.5A-D), we found DY-labeled cells in LA, dLGN, and LP (Figures 2.6B, C, F). The vast
Figure 2.6 Retrograde tracing in PVtdT mice of subcortical and cortical source neurons projecting to POR. A-C, Tangential section through deep L2/3 of flatmounted PVtdT-expressing (red) cortex, shows DY injection site (false colored blue) in POR (A). Overexposed (see artificially large injection site) black/white image showing DY labeled neurons (white dots) in LA and multiple cortical areas segmented according to Gămănuț et al., (2018) (B). Overlay of DY-labeled neurons with PVtdT expression (C). D-F, Coronal sections showing DY-labeled neurons in shell of dLGN (D), LPLR, LPMR (E), and LA (F).
majority of thalamic inputs originated from different parts of the LP (LPLC, LPLR, LPMR) (Figures 2.6D-E). Inputs from the dLGN were sparse, but we found consistently DY-labeled cells in the shell (Figure 2.6D), suggesting that POR receives visual input from both the direct dLGN→POR and the indirect SC→LP→POR\textsuperscript{12} pathway. Limbic cortex contained DY-labeled cells in LA, ENTm and ENTl (Figures 2.2B, C), all of which receive strong inputs from POR (Figures 2.3E’, 2.S1E, Wang et al., 2012\textsuperscript{29}), suggesting bidirectional communications between POR, amygdala, and entorhinal cortex. DY-labeled POR-projecting neurons were also found in multiple neocortical areas including: V1, PM (posteromedial), MM (mediomedial), RSP (retrosplenial), AM (anteromedial), RL (rostromedial), AL (anterolateral), LM, LI, P, ECT, TEp, TEa (temporal anterior), DP (dorsal posterior), AUD, Alv (anterior insula, ventral part), GU (gustatory), ORBl (orbitofrontal lateral part), ILA (infralimbic anterior part), and ACAv (anterior cingulate ventral part) (Figures 2.6A-C).

**Cell bodies and apical dendrites of POR→amygdala and POR→ENTm projecting neurons are aligned with M2- interpatches**

The striking specificity of amygdala→POR axonal projections for M2- interpatches suggested that these inputs may target L1 apical dendrites of long-range projecting neurons. Of particular interest were POR neurons projecting to downstream targets such as the amygdala and ENTm, known for their roles in affective processing\textsuperscript{49}, memory, and spatial navigation\textsuperscript{50}, respectively. To label the complete dendritic arbor of such neurons, we performed retrograde viral tracing from LA/BLA and ENTm in Ai9 mice with AAV2retro-CAG-Cre. M2+ patches were identified by immunostaining for M2 and dividing fluorescence intensity into quantiles. The results show that LA/BLA and ENTm injections retrogradely labeled pyramidal cell dendrites, cell bodies, and
Figure 2.7 Distribution of retrogradely labeled apical dendrites of POR→amygdala and POR→ENTm projecting neurons in L1 of POR. A-C, Tangential section through flatmounted POR showing patchy expression of M2 immunostaining in L1 (A) and non-uniform branching pattern of apical dendrites (false colored green, arrows,) in
L1 of POR→ENTm projecting neurons labeled by retrograde tracing with rAAV2-Retro/CAG-Cre in Aδ9 mice (B). Overlay of A and B shows that dendritic branches (false colored green) are preferentially associated with M2- interpatches (C). D, Tracings of retrogradely labeled dendrites (green) in L1 of POR overlaid onto contour plot of M2 expression. Shades of gray represent 6 quantiles of fluorescence intensity. E-G, Tangential section through flatmounted POR showing patchy expression of M2 immunostaining in L1 (E) and non-uniform branching pattern of apical dendrites (false colored green, arrows) of POR→LA/BLA projecting neurons labeled by retrograde tracing with rAAV2-Retro/CAG-Cre (F). White arrows indicate dendrites aligned with the cortical surface. Red arrow points to a thick vertically ascending dendritic trunk which issues terminal branches at the L1/2 border. Because the section is not perfectly parallel to L1 and cuts into L2 it shows a retrogradely labeled cell body at the L1/2 border (arrowhead). Overlay of E and F shows that dendritic branches (green) and cell bodies at the L1/2 border are preferentially aligned with M2- interpatches (G). H, Length of dendritic branches of POR→ENTm and POR→LA/BLA projecting neurons in M2+ patches M2- interpatches of L1 of POR. The fraction of total dendritic lengths for both types of neurons shows a significant (Pearson correlation) bias for M2- interpatches. I, J, Laminar distribution (mean ± SEM) of retrogradely labeled cells in POR, PORa, LM, LI, and P projecting to ENTm (I) and LA/BLA (J), respectively.
their local axonal projections in POR (Figures 2.7A-C, D-F), PORa, P, ECT, TEP, LM, and LI (Figures 2.7I, J). Dendrites were readily distinguished from thin, tubular axons (≤ 0.2 µm in diameter) by the tapered morphology, greater thickness (0.4-1.5 µm in diameter), and the presence of spines. Module preference of apical dendrites in L1 was quantified by manually tracing branches in 3-4 select regions, illustrated by the example shown in Figure 2.7D. Most strikingly, we found that apical dendrites of POR→LA/BLA-projecting cells branched preferentially in M2- interpatches of L1 (2.7E-G). Similar results were obtained for POR→ENTm projecting neurons (Figures 2.7A-C). Comparison of total dendrite lengths in M2 intensity quantiles showed a strong negative correlation between M2 intensity and labeled dendrites (POR→LA/BLA; R = -0.93, P < 10^{-10}, N = 4 mice; POR→ENTm; R = -0.99, P < 10^{-8}, N = 2 mice; Pearson correlation), supporting the finding of spatial clustering in M2- interpatches (Figure 2.7H).

Somas of retrogradely labeled POR→ENTm (2 mice) and POR→LA/BLA (2 mice) cells were found in L2-6 of POR (Figures 2.7I, J). Similar size injections into ENTm and LA consistently labeled more cells in POR than in any other area. LM→ENTm and LI→ENTm neurons were mostly confined to L2/3. P→ENTm, PORa→ENTm and POR→ENTm cells were biased to deep layers with a preference for L5/6 border (Figure 2.7I). Deep layers were also the preferred source of cells projecting to LA/BLA. Unlike POR→ENTm cells POR→LA/BLA neurons were distributed across the thickness of L5 (Figure 2.7J).
2.6 DISCUSSION

*Postrhinal cortex contains two modularly organized areas*

We have found that postrhinal cortex contains two distinct areas, POR and PORa. Both areas have granular cytoarchitectures, are contained within a M2+ region, and have sharp borders with the surrounding M2- areas of ECT and TEa. We found no support for a third area, PORv, which based on its agranular cytoarchitecture and lack of M2 expression more likely corresponds to rodent ECT or primate TH. The POR/PORa border was revealed by a transition from the orderly visuotopic map in POR to the more dispersed but spatially clustered connectivity in PORa. A similar map reversal exists between primate areas TFO and TF, suggesting that the areal organization of the PV- and M2-expressing parahippocampal cortex in rodents and primates may be homologous.

A striking feature of POR, PORa, LM, and LI is the nonuniform tangential distribution of M2+ patches and M2- interpatches in L1. Patchiness is a well-known attribute of primate extrastriate cortex and has also been shown to exist in the intrinsic connectivity of TF. The patchiness in POR, PORa, LM, and LI resembles the pattern we have seen in mouse V1. However, unlike the quasi-isotropic modules in V1, patches in higher visual areas were anisotropic, and showed aspect ratios similar to the overall shape of the area in which they reside. Additionally, we found a reduced (relative to V1) magnification factor per patch in LM (12.5% smaller), POR (37.5% smaller), and LI (50% smaller), indicating that a module in each of these areas represents a larger portion of visual space than in V1. The results further suggest that in ascending through the hierarchy of V1, LM, LI, and POR the point image at the top level is represented by the lowest number of modules, which indicates that inputs from diverse retinotopic locations.
Figure 2.8 Circuit diagram of inputs from the dLGN, LP, and amygdala (Amy) to M2+ patches (red) and M2-interpatches (pink) in POR. Apical dendrites and cell bodies of POR neurons projecting to Amy and ENTm (red) are aligned with M2- interpatches. Projection targets of neurons (black) in patch modules are unknown.
converge and that individual patches may integrate a broader set of stimulus attributes.

**POR contains separate module-specific circuits with dLGN, LP, amygdala, and ENTm**

Our anterograde tracings with BDA have shown that only the ventral stream areas LM, LI, P, and POR provide input to the amygdala. No such connections were found from V1 and the dorsal stream areas AL, RL, A, AM, and PM. Conceptually similar results were reported earlier in rat55. Together the results suggest that only higher visual areas involved in the perception and identification of objects and the context in which they appear have direct connections to the amygdala, while areas which play a role in visually guided actions do not56. From this simplified perspective it is easy to overlook that POR is highly interconnected with dorsal and ventral stream areas29,31. Through these connections POR combines information across streams, associates objects with locations (i.e. context10) and, through reciprocal interactions with the amygdala, assigns affective credits to neurons which guide behavior19,57. Notably, only POR sends significant input to the BLA nucleus, which projects onward to the CeA58 and is the main driver of behavioral responses from the amygdala59. Thus, it appears that POR has more direct control over visually-related affective behavior than areas LM, LI, and P, whose projections are weak and largely confined to LA.

The patchy pattern of M2 expression we have found in L1 of areas LM, LI, POR, and PORa reveals that the modular organization of V127 is preserved across higher visual areas of the ventral stream29. Modularity, in the sense used here, refers to the columnar organization of stimulus-selective responses and the spatial clustering of projection neurons within an area60. That such constraints emerge from the segmentation of the plexiform sheet of L1 has only been
shown in V1, where a patchy pattern of M2 expression labels sites of thalamocortical and intracortical synaptic input to apical dendrites of pyramidal neurons\textsuperscript{27,28}. We have found that similar patchy patterns in L1 organize the modularity of amygdalocortical and thalamocortical inputs to POR, PORa, LM, and LI. The results show that of all layers the LA innervates L1 most strongly and preferentially targets M2- interpatches. Thalamic inputs from dLGN and LP to POR, PORa, LM, and LI not only differ from amygdalocortical afferents in their laminar distribution but in their preferential targeting of M2+ patches in L1. The distinctive connectivity patterns suggest that amygdalocortical and thalamocortical inputs to L1 target apical dendrites of separate populations of POR and PORa neurons, whose cell bodies are located in the layers below. Spatial clustering of pyramidal cell dendritic tufts in L1 are well-known features of rat V1 and retrosplenial cortex\textsuperscript{61,62}. Here, we demonstrate directly that the apical dendrites of POR→amygdala projecting neurons preferentially terminate in M2- interpatches. Thus, it is attractive to speculate that pyramidal cells in POR with dendrites in M2- interpatches may be modulated by affective information from the amygdala\textsuperscript{19,63}, which are driven directly by visual input from LP\textsuperscript{23}. By contrast, responses of POR neurons with dendrites in M2+ patches may be influenced preferentially by direct visual inputs from the dLGN and LP, rather than by inputs ascending through the hierarchical cortical network originating in V1\textsuperscript{9,12,26}.

The overlap between dendrites of POR output neurons with inputs from the amygdala suggests that the amygdala and POR are interconnected through a reciprocal loop. However, overlap does not demonstrate synaptic connectivity and leaves unanswered whether the reward-modulated responses recorded in POR\textsuperscript{19} are mediated by synaptic contacts in L1. Tagging sensory stimuli with affective significance imported from the amygdala is not the only influence on the
multimodal response profile of POR. POR neurons are also sensitive to the spatial context in which objects appear\textsuperscript{10}. This information may derive from dorsal stream inputs\textsuperscript{29,31} and is sent to multiple downstream targets\textsuperscript{18} including the ENTm, with which POR is reciprocally connected (Figures 2.6B-C, 2.7E-G). We have found that, similar to POR→amygdala projecting cells, the apical dendrites of POR→ENTm neurons terminate preferentially in M2- interpatches. These dendrites may belong to POR→LA/BLA and POR→ENTm cells at different depths of L5 or belong to the same neurons with branching projections to both the amygdala and ENTm. Regardless, the results suggests that some POR neurons with dendrites in M2- interpatches send affect-modulated outputs to ENTm where they may enhance the emotional salience of landmark information, unexpected objects in the external world\textsuperscript{14}, and defensive responses to visual threats\textsuperscript{23,57}.

In contrast to amygdala→POR projections, we found that dLGN→POR and LP→POR inputs overlap with M2+ patches of L1. Retrograde labeling from POR confirmed that dLGN inputs originate from matrix-type shell neurons, which are known to receive afferents from the retina and the superior colliculus (SC)\textsuperscript{64–67} and, like the SC→LP→POR pathway, convey visual motion information to its target\textsuperscript{12,68}. Inputs from matrix-type thalamic neurons to dendrites in L1 have been shown to elicit spiking in pyramidal cells of rat barrel cortex\textsuperscript{69} and may possess similar synaptic strength in POR. Both pathways carry locomotion signals, in which running speed is either synchronized or desynchronized with optic flow motion\textsuperscript{70}. Synchronous convergent thalamocortical inputs, heavily biased from LP, to M2+ patches in POR may mostly signal moving objects at speeds different from the animals’ own motion. These direct thalamic inputs to apical dendrites in M2+ patches of POR may increase the sensitivity of POR neurons\textsuperscript{71} to non-
threatening, moving stimuli independent of the hierarchical cortical circuitry\textsuperscript{12}. Future studies are necessary to determine to which circuit M2+ patch-aligned POR cells belong (Figure 2.8).

2.7 ABBREVIATIONS

Anatomical nomenclature from: Dong (2008)\textsuperscript{72}, Franklin and Paxinos, (2007)\textsuperscript{73}

ABC- Avidin Biotin Complex
AOB - Accessory olfactory bulb
A - Anterior area
AM - Anteromedial area
ACAd, ACAv - Anterior cingulate area dorsal, ventral
AId, Alv, Alp - Agranular insular area dorsal, ventral, posterior
Amy - Amygdala
AOB - Accessory olfactory bulb
APTD - Pretectal nucleus, anterodorsal
AUD, AUDp, AUDpo, AUDv - Auditory cortex primary, posterior, ventral
BDA - Biotinylated dextran amine
BLA - Basolateral amygdala
BMA - Basomedial amygdala
Ce, CeA, CeL, CeM - Central amygdala, anterior, lateral, medial
Chrm2, Chrm2-tdT - Muscarinic acetylcholine receptor M2, tdTomato
CL - Centrolateral thalamic nucleus
CLA - Claustrum
CP, CPu - Caudoputamen
DEn - Endopiriform nucleus, dorsal
DY - Diamidino Yellow
LGN, dLGN - Lateral geniculate nucleus, dorsal
DP - Dorsal posterior area
DLPAG - Periaqueductal gray, dorsolateral
ECT - Ectorhinal area
eml - external medullary lamina
ENTl, ENTm - Entorhinal area lateral, medial
EP - Endopiriform nucleus
FRP - Frontal pole
GU - Gustatory area
Hip - Hippocampal formation
IGL - Intergeniculate leaflet
ILA - Infralimbic area
IMA - Intramedullary thalamic nucleus
int - Internal capsule
LA - Lateral amygdala
LM - Lateromedial area
LLA - Laterolateral anterior area
LI - Laterointermediate area
LP - Lateral posterior nucleus
LPLC - Lateroposterior nucleus, laterocaudal
LPMC - Lateroposterior nucleus, mediocaudal
LPLR - Lateroposterior nucleus, laterorostral
LPMR - Lateroposterior nucleus, mediorostral
LDDM - Laterodorsal nucleus, dorsomedial
LDVL - Laterodorsal nucleus, ventrolateral
M2 - M2 muscarinic acetylcholine receptor
MDL - Mediodorsal thalamic nucleus, lateral
MG, MGD, MGV - Medial geniculate nucleus, dorsal, ventral
MM - Mediomedial area
MO, MOp, MOs - Motor cortex, primary, secondary
MZMG - Medial geniculate nucleus, marginal zone
OB - Olfactory bulb
OT - Olfactory tubercle
ORBl, ORBm - Orbitofrontal area lateral, medial
PERI - Perirhinal area
P - Posterior area
PF - Parafascicular thalamic nucleus
Pir - Piriform cortex
PL - Prelimbic area
PM - Posteromedial area
Pn - Pons
Po - Posterior thalamic nucleus
POR, PORa, PORv - Postrhinal area, anterior, ventral
PPT - Pretectal nucleus, posterior
PV, PVtdT - Parvalbumin, parvalbumin tdTomato
RSP - Retrosplenial area
RL - Rostrolateral area
Rt - Reticular thalamic nucleus
SCs - Superior colliculus superficial layers
SCi - Superior colliculus deep layers
SSp - Somatosensory cortex primary
SSs - Somatosensory cortex secondary
SubG - Subgeniculate nucleus
TEa - Temporal area anterior
TEp - Temporal area posterior
TF, TFO, TH - Primate parahippocampal areas
VA - Ventral anterior thalamic nucleus
VL - Ventral lateral thalamic nucleus
vLGNm, vLGNp - Ventral lateral geniculate nucleus, magnocellular, parvocellular
VPL, VPM - Ventral posterior thalamic nucleus, lateral, medial
V1 - Visual cortex primary
ZI - Zona incerta

2.8 REFERENCES


Chapter 3: Interdigitating Modules for Visual Processing During Locomotion and Rest in Mouse V1

3.1 ABSTRACT

Sensory responses are modulated by behavioral state. In visual cortex, locomotion elicits a state change of increased firing rates and response gain, as a means of improving the fidelity of stimulus representation\(^1\). It is not known whether this state change occurs uniformly across visual cortex or whether it is a feature of a specific subpopulation of cells. Using calcium imaging in awake mice, we find periodic clusters of neurons in Layer 2/3 with high locomotion modulation which are aligned with regions of low muscarinic acetylcholine receptor 2 expression (M2-interpatches). M2-interpatch cells showed an increase in correlated trial-to-trial variability (noise correlations) at long distances, suggesting a role in integrating stimuli across the visual field, possibly for detecting optic flow or large moving objects. We find that two network architectures may account for this population-specific locomotion modulation: increased expression of somatostatin (SST) projections in M2-interpatches and preferential targeting by secondary motor cortex (MOs) inputs to M2-interpatches. Finally, we elucidate a possible role of M2-interpatch cells in visual guidance of behavior by showing that somas and dendrites aligned with M2-interpatches project to primary motor cortex (MOp). These findings reveal a specialized role for M2-interpatch cells in integrating and exploiting stimuli from a wide visual field during locomotion.
3.2 INTRODUCTION

Sensory responses are not rigid representations of stimuli, but instead are modulated by behavioral context. A prominent example of this occurs in primary visual cortex (V1), in which locomotion increases the gain of visual responses in excitatory neurons\textsuperscript{2,3}. This increase in responsiveness has been shown to increase the fidelity of stimulus representations across the neuronal population\textsuperscript{1}, enhance plasticity\textsuperscript{4}, improve visual object detection\textsuperscript{5}, and facilitate recovery of visual function after cortical damage\textsuperscript{6}. Multiple mechanisms driving this modulation have been described, including release of inhibition from SST neurons\textsuperscript{7–9} and activation of long range inputs from the midbrain locomotor region\textsuperscript{10}.

The wide variability of locomotion modulation among V1 neurons\textsuperscript{11} raises the question of whether modulation is randomly distributed among cells, or is present in a discreet population distinguished by connectivity, response tuning, or location. We addressed this question by measuring locomotion modulation in cells aligned with clusters of high and low expression of muscarinic acetylcholine receptor 2 (M2) in layer 1 (M2+ patches and M2- interpatches). Previously we showed that Layer 1 (L1) contains interdigitating modules of M2-rich and M2-poor patches\textsuperscript{12}. L2/3 cells aligned with M2- interpatches are tuned for features of moving objects, including temporal frequency and motion coherence, suggesting that they may play a specialized role in processing the dynamic visual stimuli encountered during locomotion.

We investigated possible anatomical organizations for differential locomotion modulation in M2 modules. Because inhibition from SST interneurons plays a central role in locomotion modulation of visual responses\textsuperscript{7}, we tested SST expression in M2+ patches and M2- interpatches.
A recent study demonstrated that MOs provides a motor efference copy signal to V1, motivating us to determine whether MOs→V1 projections preferentially target one of the M2 modules. Finally, we performed retrograde viral tracing of projections from V1 to MOp, to determine whether motor cortex-projecting cells align with M2+ patches or M2- interpatches.

The results show that locomotion modulation is stronger in M2- interpatches than M2+ patches and that locomotion-tuned M2- interpatch cells have increased correlations in trial-to-trial variability at long distances. We found that M2- interpatches overlay with inputs from local SST cells and feedback inputs from MOs, and provide the majority of outputs to MOp. These results demonstrate that locomotion modulation is a specific feature of M2- interpatch cells, which may play a role in processing stimuli from across the visual field to guide behavior during self-motion.

3.3 RESULTS

Locomotion modulation of visual responses is greater in M2- interpatch cells

We injected AAV1.hSynapsin.Flex.GCaMP6f into multiple sites of V1 of adult Chrm2tdT x Emx1-Cre mice to express GCaMP6f in excitatory pyramidal neurons. Chrm2tdT mice express fluorescently tagged M2, enabling ex vivo detection of M2+ patches, to be overlaid with recorded cells. A headplate was implanted over a cranial window for access to the injection sites (Figure 3.1A). Neuronal activity of L2/3 pyramidal cells was monitored as calcium responses in a single tangential plane via 2-photon microscopy. Awake mice were head-fixed, placed on a running wheel, and allowed to locomote spontaneously while three blocks of visual stimuli were presented (Figure 3.1B). First, circular drifting gratings were presented at various points in a 4x4
grid across the monitor, and cell responses were used to compute the average receptive field of hundreds of cells. Next, the stimulus screen was turned off for 10 minutes, during which spontaneous activity of cells was recorded in total darkness. Third, a 30-degree diameter drifting circular grating was presented with a range of spatial frequencies (SF; 0.01–1.6 c/deg), temporal frequencies (TF; 0.1-12Hz), and orientations (OS; 51° increments). After recording up to four sessions in separate non-overlapping cortical locations per mouse (N = 9 mice), mice were sacrificed and cortex was flatmounted and tangentially sectioned. L1 Chrm2tdT expression was imaged with epifluorescence microscopy. M2 images were high-pass filtered, blurred, and divided into six intensity quantiles, the top 3 of which were considered M2+ patches and the bottom 3 M2- interpatches (see Methods). In vivo recorded cells were aligned with their location in ex vivo sections (Figure 3.1C-H; see Methods), from which it was determined which M2 quantile they aligned with.

For each cell, a locomotion modulation index (LMI)\(^\text{14}\) was computed as \((R_L - R_S)/(R_L + R_S)\), where \(R_L\) is the mean \(\Delta F/F\) stimulus response during locomotion trials (> 0.1cm/s), and \(R_S\) is the mean response during stationary trials (< 0.1cm/s running speed). Figure 3.2A shows SF, TF, and OS tuning curves computed during stationary (black curves) and locomotion (red curves) trials for example cells near the centers of M2- interpatches (quantiles 1-2), near the centers of M2+ patches (quantiles 5-6), or in the transition regions between M2 modules (quantiles 3-4). The inset in Figure 3.2A (upper left) shows the stimulus response timecourse of an example M2-interpatch cell to its preferred spatial frequency, for which the response during locomotion trials (N=4 trials) was approximately twice that of its response to stationary trials (N=6 trials). Most locomotion-tuned cells showed a positive gain (753 positive, 145 negative; average LMI =
Figure 3.1 Physiology and alignment protocol. A, Injection sites of AAV2/1.hSyn.GCaMP6f in V1 after window implantation, visualized by overlaying brightfield image with GFP expression image. Inset: ex-vivo tangential section showing remaining GCaMP expression overlaid with ChrM2tdT expression. B, Stimulation protocol. 1. Receptive fields were mapped by presenting drifting circular square wave gratings in one of sixteen grid locations on the screen per trial. 2. All visual stimuli were removed and cells were recorded in darkness for 10 minutes. 3. Drifting gratings were presented with varying spatial frequency, temporal frequency, and orientation to obtain tuning curves for each cell. C-H, Alignment of recorded cells with M2+ patches. C, Time-averaged in vivo GCaMP signal from a recorded plane showing somas of active cells. D, Ex vivo GCaMP expression from tangential section in the same location as the recording plane, after perfusion and sectioning. E, Overlay of ex vivo GCaMP expression from D aligned with image of ex vivo ChrM2tdT expression in L1, including M2+ patches. F, ChrM2tdT expression image from E after high-pass filtering and blurring. G, ChrM2tdT image from F with borders between 6 intensity quantiles drawn. H, In vivo GCaMP image from C overlaid with M2 quantiles from G, allowing cells from in vivo recordings to be assigned an M2 quantile. M2+ patches shaded violet.
Figure 3.2 Locomotion modulation and orientation tuning in M2+ patches and M2- interpatches. A, Tuning curves from example cells for spatiotemporal stimulus parameters. Red traces illustrate tuning curves generated by trials with locomotion (>0.1cm/sec) while black curves were generated from trials with no locomotion. The leftmost column contains M2- interpatch cells (quantiles 1-2), the rightmost contains M2+ patch cells (quantiles 5-6), and the middle column contains cells near the border between M2+ patches and M2- interpatches (quantiles 3-4). Fitted tuning curves are presented with dots illustrating responses from individual trials. Inset: trial-averaged timecourses of a sample M2- interpatch neuron to its preferred spatial frequency stimulus for trials during locomotion (red) or when stationary (black). Errorbars show SEM at each time bin. B, Distributions of locomotion modulation of visual responses in M2+ patch (quantiles 4-6) and M2- interpatch (quantiles 1-3) cells. Horizontal bars show group means. Left, locomotion modulation of cells whose receptive fields were covered by the grating stimulus (P<0.01, t-test). Right, locomotion modulation of cells for which the stimulus was in the surround and did not stimulate the cell’s receptive field (P>0.05, t-test). C, Orientation selectivity index (OSI) by M2 quantile of cells whose receptive fields were covered by the grating stimulus. OSI was computed from responses during stationary and locomotion trials combined. Error bars show SEM in each quantile. D, Orientation selectivity index by M2 quantile of cells for which the stimulus was in the surround and did not stimulate the cell’s receptive field. Error bars show SEM in each quantile.
0.17±0.01) in response to each of the 3 types of stimuli (SF, TF, OS) presented in the centers of the receptive field during locomotion trials. When the analysis only included cells whose receptive field centers were covered by all 3 types of stimuli, M2- interpatch cells showed a 48.6±8% greater LMI than M2+ patch cells (Figure 3.2B; N = 9 mice; N=283 cells; P<0.01, t-test). In order to test whether this module-specific difference in LMI was due to inputs targeting the receptive field center of recorded cells or was an effect that also involved the surround, we compared the LMI of responses to large stimuli that spared the center. In such surround-stimulated cells, we found that there was no difference between M2+ patch cells and M2-interpatch cell in LMI computed from the combined responses to SF, TF, and OS (N=615 cells, P=0.61, t-test). Center-stimulated cells were also 2.4-times more responsive on combined locomotion and stationary trials than surround-stimulated cells when presented with preferred orientation gratings (Figure 3.S1I). Locomotion did not affect the average tuning width (half-width at half-maximum, HWHM) of cells for any stimulus parameter (Figure 3.S1A-C).

**Orientation selectivity is stronger in M2+ patches**

Because a previous study on V1 modules in anesthetized mice found that M2+ patches appear to be specialized for object shape12, we asked whether orientation tuning was differentiated by M2 module. Strength of orientation tuning was measured by the orientation selectivity index (OSI), computed as $(R_{\text{pref}} - R_{\text{ortho}})/(R_{\text{pref}} + R_{\text{ortho}})$, where $R_{\text{pref}}$ is the average ∆F/F response of the cell to its preferred orientation, and $R_{\text{ortho}}$ is its response to the orientations shifted 90° away from the preferred orientation. We found that OSI was positively correlated with M2 quantile, indicating that M2+ patch cells were more strongly tuned for orientation than M2- interpatch cells (Figure 3.2D; N=608 cells; R=0.11, P<0.01, Pearson correlation). We did not find differences in
Figure 3.51 Effects of locomotion, M2 quantile, and center/surround targeting on stimulus responses. A-C, Half-width at half maximum (HWHM) of spatial frequency, temporal frequency, or orientation tuning computed from locomotion (mean forward velocity >0.1 cm/s) and stationary trials (mean forward velocity <0.1 cm/s). For each cell, a tuning curve while stationary or during running were (t-test). Locomotion does not change the HWHM of any stimulus parameter (P>0.05, t-test). D-F, M2 quantile plotted against HWHM of spatial frequency, temporal frequency, and orientation tuning. M2+ patches and M2-interpatches did not show differences in HWHM for any tuning parameter (P>0.05, Pearson correlation). G-H, M2 quantile plotted against peak spatial and temporal frequency. M2+ patches and M2-interpatches did not show differences in peak spatial or temporal frequency (P>0.05, Pearson correlation). I, Mean responses to preferred orientation in center-stimulated vs. surround-stimulated cells. Responses were computed from locomotion and stationary trials as the mean ∆F/F during stimulus minus mean ∆F/F during the 4-second prestimulus period. Responses were greater in center-stimulated than surround-stimulated cells (P<10⁻⁸, t-test).
HWHM of spatial frequency, temporal frequency, and orientation between M2+ patches or M2-interpatches (Figure 3.S1D-F). Peak spatial frequency and temporal frequency also did not differ between M2+ patches and M2- interpatches (Figure 3.S1G-H).

**M2+ patch pairs have increased activity correlations in the absence of stimuli**

Because M2+ patches and M2- interpatches were previously shown to have distinct thalamic and cortical inputs and receive different levels of local inhibition\textsuperscript{12,15}, we asked whether pairwise activity correlations (see Methods) were distinct between the two module types. Spontaneous correlations were computed as the Pearson correlation coefficient between the ∆F/F traces of two cells throughout the dark period, during both stationary and locomotion epochs. Pairs were divided into those consisting of two M2+ patch cells, two M2- interpatch cells, and mixed pairs. Previous studies of activity correlations in mouse V1 found that pairwise correlations are reduced with increasing physical distance\textsuperscript{16,17}, so for all analyses we grouped cells by distance, using 25µm-wide bins. We first examined pairwise ∆F/F Pearson correlation coefficients during the 10-minute dark period (bin size 0.33s). We found a striking difference between pair types: in every bin from 0-275µm, M2+/M2+ pairs were more highly correlated than both mixed pairs and M2-/M2- pairs (Fig 3A; asterisks indicate significant differences (t-test) between M2+/M2+ and M2-/M2- pairs within each bin). This difference between pair types was not found in pairs at distances greater than 275µm.

**Long-distance M2- interpatch pairs have increased response noise correlations**

We next computed the correlated variability of stimulus responses (see Methods), dividing pairs
Figure 3.3 Pairwise correlated activity of L2/3 M2+ patch cells and M2- interpatch cells in V1. Pairs were divided into those containing two M2+ patch cells (violet), two M2- interpatch cells (green), and one of each type (black). A, Correlation coefficient of ΔF/F signals (mean±SEM) of cell pairs in darkness at different distances. Asterisks indicate significance level of difference between correlation coefficients in M2+/M2+ and M2-/M2- pairs within each distance bin (t-test; ⁎=p<10^{-2}, ⁎⁎=p<10^{-3}, ⁎⁎⁎=p<10^{-4}). B, Coefficient of response noise correlation (mean±SEM) between cell pairs during visual stimulation at different distances. Asterisks same as in A. C, Comparison of response noise correlations (mean±SEM) between cell pairs in which both cells were locomotion-responsive and separating distance was >275µm. Asterisks indicate significance level (t-test with Bonferroni correction; ns=not significant, ⁎=p<10^{-2}, ⁎⁎=p<10^{-3}, ⁎⁎⁎=p<10^{-4}). D, Comparison of response noise correlations (mean±SEM) between cells pairs in which neither cell was locomotion responsive and separating distance was at least 275µm. Asterisks same as in C.
by module and distance. Response noise correlations were computed by finding the Pearson correlation between response magnitudes to every trial with identical stimulus spatiotemporal features (identical SF, TF, and OS), then averaging this value across all unique stimuli\textsuperscript{18}. We found that unlike activity correlations in darkness (<0.1 cd/m\textsuperscript{2}), response noise correlations show almost no module-specific differences in any bins from 0-225\(\mu\)m. Instead, cell pairs diverged by module type at longer distances, with long-range M2-/M2- showing increasing correlation with distance and long-range M2+/M2+ and mixed pairs continuing to decrease correlation values with increasing distance. Because of our previous findings on locomotion modulation and studies reporting that locomotion affects pairwise correlations\textsuperscript{11}, we compared the response noise correlations of cell pairs whose activity was either responsive or non-responsive to locomotion. Locomotion-responsive pairs were considered to be those for which both cells showed a significant correlation between \(\Delta F/F\) and running speed (Pearson correlation, \(P<0.05\)), while non-responsive pairs were those for which neither cell’s \(\Delta F/F\) was significantly correlated with running speed. We found that among long-range locomotion-responsive pairs (distance >275\(\mu\)m), pair type played a significant role in response noise correlations (Figure 3.3C), with M2-/M2- pairs (\(N=186\)) having 67\% greater correlation coefficients than M2+/M2+ pairs (\(N=203; \ P<10^{-4}\), t-test with Bonferroni correction) and 39\% greater correlation coefficients than mixed pairs (\(N=410; \ P<10^{-3}\)). Among long-range locomotion-nonresponsive pairs (Figure 3.3D), M2-/M2- pairs (\(N=352\)) did not show the same relative increase over either M2+/M2+ (\(N=393; \ P=0.13\)) or mixed pairs (\(N=761; \ P=0.74\)). This finding suggests that increased long-range correlations between M2- interpatch cells is locomotion-specific.
Layer 1 somatostatin expression is localized to M2- interpatches

Next, we investigated possible anatomical underpinnings for the observed differences in locomotion modulation between M2+ patches and M2- interpatches. Guided by previous studies demonstrating that SST innervation in V1 plays a key role in effecting locomotion modulation of pyramidal cells\textsuperscript{7,19}, we performed M2 immunostaining in SST-Cre x Ai9 mice (SSTtdT), in which SST cell bodies, axons, and dendrites express tdTomato. Adult mice were sacrificed and perfused, and cortex was flatmounted and sectioned tangentially at 40µm. Immunostaining was then performed with an antibody against M2 to confirm M2 expression in tdTomato-labeled cells with an Alexa 647-tagged IgG. Overlaying SSTtdT and M2 expression revealed that SST expression is nonuniform in L1, with a strong preference for M2- interpatches (Figure 3.4A-C). Dividing images into M2 intensity quantiles revealed that SST expression is negatively correlated with M2 expression, with 4.6-times higher SST expression in the center of M2-interpatches than M2+ patches (Figure 3.4G; N=5 mice; P<10\textsuperscript{-8}, R = -0.85, Pearson correlation). As a negative control, the SST images were broken into 5µm-wide squares and shuffled, from which new SST intensity values were obtained for each quantile. The shuffled data did not show a relationship between SST expression and quantile (Figure 3.4H; P=0.53, R = -0.12). Additionally, SST expression was compared to dorsal lateral geniculate nucleus (dLGN) inputs to L1, which strongly localize with M2+ patches\textsuperscript{12,15}. Injection of anterograde viral tracer AAV2/1-hSyn-GFP into dLGN labeled dLGN\textarrow{V1} inputs that avoided regions with strong SSTtdT expression, supporting the finding that SST expression is localized to M2- interpatches and avoids M2+ patches (Figure 3.4D-F; N=3 mice).
Figure 3.4 Comparison of SST expression with M2+ patches and M2- interpatches in L1 of V1. A-C, Overlay of SSTtdT expression with M2 immunostaining in tangential sections through flatmounted V1 showing labeling in neuropil of L1. Patch borders were derived from the high-pass filtered, blurred M2 image. D-F, Tangential section through flatmounted V1. Overlay of SSTtdT expression with GFP-labeled inputs from dLGN in L1. Patch borders were derived from filtered, blurred GFP image. G, SSTtdT intensity in each M2 quantile. Error bars show SEM of SSTtdT intensity in each quantile. H, SSTtdT intensity in each M2 quantile after SSTtdT images have been shuffled.
Secondary motor cortex feedback preferentially targets M2- interpatches

Locomotion modulation signals may be transmitted to V1 by feedback projections from secondary motor cortex (MOs), which was recently shown in rats to provide efference copy signals to V1\textsuperscript{20}. We tested this possibility by injecting anterograde viral tracer AAV2/1-hSyn-GFP into MOs of Chrm2tdT mice (Figure 3.5D). In flatmounted, tangential sections, images of axons from MOs→V1 projections in L1 were overlaid with images of M2 expression to determine the strength of MOs projections to M2+ patches and M2- interpatches (Figure 3.5A-C). We found that MOs axonal projections preferentially targeted M2- interpatches, while providing weaker inputs to M2+ patches. The density of GFP-labeled terminal axon branches was negatively correlated with M2 quantile, with intensity in M2+ patch centers 54% lower than in M2- interpatch centers (Figure 3.5D; N=2 mice, P<0.05, R = -0.62, Pearson correlation).

V1 outputs to primary motor cortex are aligned with M2- interpatches

Our findings that M2- interpatches show greater locomotion modulation and receive inputs from MOs raised the question whether V1 cells aligned with M2- interpatches project to areas specialized for locomotor behavior. To address this question, we tested whether V1 projects to primary motor cortex (MOp), and whether any such projections are aligned with M2 modules. To find out, we injected the retrograde viral tracer AAV2retro-CAG-Cre into MOp in Ai9 mice (Figure 3.6H), to label cell bodies and dendrites of V1→MOp-projecting cells. dLGN inputs to V1 were also traced with AAV2/1-hSyn-GFP to use as a proxy for M2+ patches\textsuperscript{12,15}. Tangential sections were obtained through all layers of flatmounted cortex. Patch/interpatch borders were obtained from labeled dLGN inputs to L1, which were then aligned with lower layer sections by
Figure 3.5 Inputs of secondary motor cortex (MOs) to M2+ patches and M2- interpatches in L1 of V1. A-C, Tangential section through L1 of flatmounted V1. Overlay of Chrm2tdT expression (purple) with anterogradely AAV2/1-hSyn-GFP labeled axonal projections from MOs→V1 (green). M2+ patch borders were derived from Chrm2tdT image. D, Injection site of AAV2/1-hSyn-GFP in MOs (green). Purple shows Chrm2tdT expression, which was used to delineate areal boundaries. E, Expression of GFP intensity of MOs→V1 projections in each M2 quantile of L1.
Figure 3.6 Retrograde tracing of V1 neurons projecting to primary motor cortex (MOp). A–C, Tangential section through flatmounted V1 showing apical dendrites (A, arrowheads) of retrogradely labeled L5 cells overlaid (C) with anterogradely AAV-GFP labeled dLGN→V1 projections (B) to M2+ patches in V1. Arrow indicates branch point of apical dendrite (same as red arrow in I inset). D–F, Retrogradely labeled V1→MOp projection neurons in L5 (D, arrowheads) aligned (F) with anterogradely AAV-GFP labeled dLGN→V1 input to M2+ patches in L1 (E). G, Somas and basal dendrites (arrows) of retrogradely labeled V1→MOp-projecting cells in tangential section through L5. Arrowhead indicates cross section through ascending apical dendrite. H, Tangential section through L4 of flatmounted cortex showing injection site of rAAV2-Retro/CAG-Cre (green) in MOp. Section was immunostained for M2 (purple), which was used for areal delineation55. I, Ratios of total dendrite lengths in M2+ patches:M2- interpatches. Histogram shows the distribution of ratios generated by shuffling the labeled dendrite image over $10^6$ iterations. Arrow indicates the actual M2+ patch:M2- interpatch ratio from the non-shuffled dendrite image (0.37:1), falling significantly below the randomized distribution average (P<10^{-4}; permutation test). Inset: schematic of L5 projection neuron with apical dendrite branching in L1. Red arrow indicates apical dendrite branch point (same as arrows in A,C).
using blood vessels as landmarks. We found labeled V1→MO-op-projecting cells in deep L5, which were preferentially aligned with M2- interpatches (Figure 3.6D-F).

No labeled cell bodies were found in layers 1-4. However, apical spine-covered dendrites of the labeled L5 and L6 cells were found to ascend toward the pial surface, terminating in multiple branches within L1 (Figure 3.6A-C). We next sought to determine whether these dendrites were preferentially aligned with either M2+ patches or M2- interpatches. Manual tracings of retrogradely labeled apical dendrites were overlaid with M2 quantiles, which revealed that the length-density of apical dendrites of MO-op-projecting neurons was greater in M2- interpatches than in M2+ patches, with a M2+:M2- dendrite length ratio of 0.37:1. A permutation test was performed by shuffling the dendrite image and obtaining a new M2+ patch:M2- interpatch dendrite length ratio, over 10^6 iterations. Comparison of the actual ratio to the shuffled ratio distribution showed that the M2- interpatch preference of dendrite location was highly significant, as it deviated from a 1:1 ratio by more than 99.99% of all shuffled iterations (Figure 3.6I; P<10^{-4}, permutation test).

3.4 DISCUSSION

We found that V1 is divided into two spatially alternating subsystems of L2/3 cells with high and low locomotion modulation of visual responses, M2- interpatches and M2+ patches. Orientation tuning is stronger in M2+ patches than in M2- interpatches. Comparison of visual response correlations revealed that M2- interpatch cell pairs have increased long-range response noise correlations. Through viral tracing and immunostaining, we showed that M2- interpatches receive distinct inputs to L1, including somatostatin axons and feedback projections from MOs.
M2- interpatches provide outputs to MOp, as L5 cells projecting to MOp have somas and dendrites preferentially aligned with M2- interpatches.

*Locomotion modulation of visual responses is stronger in M2- interpatches*

Locomotion modulation of visual responses in L2/3 was stronger in M2- interpatch than M2+ patch cells. While locomotion modulation has been described in various cortical and subcortical areas\(^{21-25}\), our results demonstrate that within a single area, V1, locomotion modulation is not uniformly or randomly distributed, but instead is localized in a subsystem with distinct connectivity and visual response properties. Locomotion-induced gain in stimulus response provides computational advantages for visual processing, including reduction in membrane potential variability and increase in mutual information between spike rate and stimulus identity\(^{1,5}\). However, M2- interpatch cells may be more optimally tuned to respond to the types of stimuli encountered during self-motion, making it advantageous for locomotion modulation to be greater in this population. This is supported by our previous finding that M2- interpatches are particularly responsive to both motion coherence and movement speed\(^{12}\), two components of the optic flow which accompanies locomotion. Our findings align with that of Wekselblatt et al. (2019)\(^{26}\), who described a cell type with higher locomotion modulation and lower orientation tuning than other cell types, matching the properties of M2- interpatch cells.

*Orientation tuning is stronger in M2+ patches*

We found that M2+ patch cells have higher orientation selectivity than M2- interpatch cells (Figure 3.2C,D). These findings support the results of Ji et al. (2015), which described a greater proportion and selectivity of orientation-tuned cells in M2+ patches than in M2- interpatches.
Our results demonstrate that differences in spatiotemporal tuning of M2+ patch and M2-interpatch cells is present during wakeful behavior and extends the results obtained in anesthetized mice. Our finding of clustered orientation tuning also aligns with that of Kondo et al. (2016), who described a columnar organization of orientation tuning in mouse V1. Stronger tuning for orientation may indicate that the M2+ patch subsystem is specialized for detecting and identifying static shapes, rather than moving objects encountered in the context of locomotion.

*Activity correlations in M2+ patches and M2- interpatches depend on module and distance*

We measured the correlated activity of M2+ patches and M2- interpatches to determine whether these populations form separate subnetworks with increased intra-network functional connectivity. We first investigated the activity correlations between cells in the absence of visual stimuli. Short-range (<275µm) pairwise correlations in darkness between M2+ patch cells were greater than those between short-range M2- interpatch pairs (Figure 3.3A). One possible mechanism for this module-specific difference is the preferential dLGN inputs to M2+ patches. Inputs from the dLGN shell to L1 of V1 are retinotopically precise, clustered, and their axonal branches extend a short range (relative to LP inputs to L1). Thus the spontaneous activity of these inputs may drive activity correlations between neighboring M2+ patch cells without synchronizing the activity of more distantly spaced M2+ patch cells.

We measured the response noise correlations of cells from both module types to investigate shared within-network inputs that are active during visual stimulation. Locomotion-responsive M2- interpatch cell pairs showed increased response noise correlations over M2+ patch pairs at long distances (> 225µm; Figure 3.3B). It was surprising to find response noise correlations...
increasing with distance greater than 225µm, as previous reports have found that pairwise correlations decrease as distance between cells increases\textsuperscript{16,17}. Our findings do not broadly contradict this trend, as most pairs, including M2+/M2+ pairs, M2+/M2- pairs, and pairs that were not locomotion responsive exhibited reduced correlations at greater distances. Instead, our results show that locomotion-responsive M2- interpatch cells are an exception to this rule and may integrate visual information from distant locations of the visual field.

Receptive field centers of M2- interpatch cells cover about 10-15° of the visual field and do not possess larger receptive fields than M2+ patch cells.\textsuperscript{12} Long-range connections between M2-interpatch cells, reaching farther than the 250x360µm (azimuth x elevation) range of the cortical point image\textsuperscript{12}, may facilitate detecting optic flow during locomotion by integrating the responses of distant cells. An intriguing possibility is that M2- interpatch cells coordinate their activity across visual space to detect violations of expected optic flow patterns\textsuperscript{31,32}, and that these signals of unexpected movement are transmitted to M2- interpatch apical dendrites via LP inputs\textsuperscript{15,33}. Lateral projections within V1 have been shown to preferentially connect cells with shared orientation tuning\textsuperscript{34}, thus these retinotopically distant M2- interpatch pairs may also facilitate the detection of elongated edges and the directions of large moving objects encountered while running.

These increased noise correlations between highly locomotion-responsive cells may appear to contradict the finding that locomotion, similarly to spatial attention, improves discriminability by reducing noise correlations\textsuperscript{1,11,35}. However, noise correlations only reduce discriminability if they are greater between cells representing similar stimuli\textsuperscript{36}. These highly correlated M2- interpatch
cells represent different regions of the visual field, unlike most previous experiments measuring decorrelation in mouse V1 during locomotion, which recorded from cells in the same or in closely spaced cortical columns. Thus correlations between distantly spaced M2- interpatch cells which integrate information across the visual field do not necessarily reduce stimulus discriminability, because these cells are likely to represent different visual objects.

Changes in response noise correlations are indicative of behavioral state alterations produced by top-down inputs. Locomotion may thus be a behavioral state characterized in the visual cortex by altered network dynamics which are favorable to detection of particular types of stimuli. This possibility is supported by the finding that during locomotion, visual detection of targets presented globally (anywhere in the visual field) is improved relative to detection of targets presented at a fixed, local position in the visual field. Our finding of increased long-range correlations between locomotion-responsive M2- interpatch cells suggests that this population contributes to the shift toward detection of whole-field stimuli during locomotion.

**Sources of long-range M2- interpatch correlations**

What are the mechanisms driving these increased long-range correlations? We propose three possibilities. First, they may be determined by the different types of thalamic inputs targeting M2+ patches and M2- interpatches. While M2+ patch cells receive retinotopically precise dLGN inputs, M2- interpatch cells receive preferential inputs from the LP which cover a wider proportion of the visual field. M2- interpatch cells within 50μm of one another, where noise correlation is high, have overlapping receptive fields driven by shared inputs, which increase correlated activity. We suggest that as distance between cells surpasses the dimensions
of the point image, each cell’s receptive field overlaps the other cell’s surround more, leading to mutual inhibition\textsuperscript{41} and reduced correlation. At even greater distances however, their receptive fields may no longer fall in one another’s suppressive surrounds, and shared, retinotopically broader inputs from LP may become a more dominant driver of noise correlation. This explanation is supported by the finding that somatostatin inputs play an essential role in creating the suppressive surround fields of pyramidal cells\textsuperscript{42,43}, as we have shown that somatostatin inputs are stronger in M2- interpatches.

Notably, we found that the increase in long-range noise correlations between M2- interpatch pairs occurs specifically between those that are responsive to locomotion. Thus a second possible mechanism for increased M2- interpatch correlations may be the reduced surround suppression which locomotion induces in many V1 neurons\textsuperscript{44}. By making the surround field more facilitative of responses, locomotion may facilitate excitatory communication between cells in different parts of the visual field, increasing those pairs’ noise correlations.

A third possible driver of these increased long-range response noise correlations is direct long-range projections between M2- interpatch cells. M2- interpatch cells may make lateral projections which preferentially target distant M2- interpatch cells, driving noise correlations. This possibility is supported by findings in rat\textsuperscript{45} and recent retrograde and anterograde tracing results showing that long-range projections within V1 strongly favor M2- interpatches (unpublished data; Ji W, D’Souza RD, Burkhalter A).
**Somatostatin expression is localized to M2- interpatches in layer 1**

In our search for mechanisms of differential locomotion modulation in M2+ patches and M2-interpatches, we compared the expression of SST and M2 in L1. We found a striking nonuniformity of SST expression, with SST largely avoiding M2+ patches and targeting M2-interpatches (Figure 3.4). This SST expression may indicate axonal inputs from Martinotti cells in lower layers targeting the apical dendrites of M2- interpatch pyramidal cells\(^{46,47}\). Findings implicating a VIP→SST→pyramidal circuit in locomotion modulation of responses\(^{7,8}\) suggest that this preferential innervation of M2- interpatches contributes to the increased locomotion modulation of pyramidal cells residing in these regions.

**Primary and second motor cortex are preferentially connected with M2- interpatches**

We found stronger feedback projections from MOs to M2- interpatches than to M2+ patches. These feedback inputs may contribute to the greater locomotion response modulation in M2-interpatches, either by indirectly disinhibiting SST neurons via VIP neurons or by exciting apical dendrites of pyramidal cells\(^{48}\). MOs feedback to V1 provides signals related to heading and optic flow\(^{13,20}\), suggesting that these inputs to M2- interpatch cells tune their responses for visual processing during navigation. Clustering of heading selectivity and perception-related activity are a known property of neurons in macaque multisensory medial superior temporal and ventral intraparietal areas\(^{49}\), suggesting that in mouse these functions are carried out at a lower stage of the cortical hierarchy\(^{50}\).

We traced projections of V1 M2+ patches and M2- interpatches to MOp. Dendrites and cell bodies of L5 V1 neurons projecting to MOp align with visual motion-preferring M2-
interpatches more frequently than with M2+ patches. Labeled L5 cells are likely direction- and
temporal frequency-selective cortico-cortically or subcortically projecting neurons with apical
dendrites extending to L1, where they receive self-motion inputs from anterior cingulate and
presumably retrosplenial cortex. The finding of L5 projection neurons preferentially aligned
with M2- interpatches expands the former concept of M2 modules in mouse, as it demonstrates
that M2+ patches and M2- interpatches functionally extend below L2/3 into deeper cortical
layers.

Summary
Overall, our findings suggest that V1 contains two populations of excitatory neurons with regard
to locomotion modulation, the highly modulated M2- interpatch cells and the weakly modulated
M2+ patch cells. M2- interpatch cells’ locomotion modulation is likely driven by increased
VIP→SST→pyramidal cell inputs and feedback from MOs, which may also provide efference
copy signals. M2- interpatch cells integrate information from distant locations of the visual field,
possibly to detect optic flow or large moving objects, and send projections to MOp to facilitate
visually guided behavior. M2+ patch cells are more strongly tuned for orientation and show
stronger pairwise correlations at shorter distances, possibly for the episodic detection of
landmarks used to update path integration coordinates during.

3.5 MATERIALS AND METHODS

Animals
We performed experiments using 5-10 week-old female and male Chrm2tdT-D knock-in
(BG6.Cg-Chrm2tm1.1Hze)/J x Emx\textsuperscript{ires-cre}, Chrm2tdT-D knock-in (BG6.Cg-Chrm2tm1.1Hze)/J,
Sst-IRES-Cre x Ai9 (Gt[ROSA]26Sortm9(CAG-tdTomato)Hze)/J, and Ai9 (Gt[ROSA]26Sortm9(CAG-tdTomato)Hze)/J mice. Experimental procedures were performed in accordance with the National Institutes of Health guidelines and under the approval of the Washington University Institutional Animal Care and Use Committee.

**General surgical procedures**

Mice were anesthetized with intraperitoneal injections of a mixture of ketamine (86mg/kg) and xylazine (13mg/kg). Buprenorphine-SR (0.1 mg/kg, SubQ) was injected prior to surgery for analgesia. Mice were head-fixed on a stereotactic apparatus. Body temperature was monitored and maintained at 37°C. Viral injections were delivered via glass micropipettes (tip diameter 20µm) attached to a Nanoject II pump. For all cortical injections, two injections were made, at 0.3mm depth and 0.5mm depth below the pial surface. Pipettes were kept in place for 5 min after each injection to allow for diffusion into the tissue. In cases where a window and head plate were not implanted, the scalp was stapled and secured with wound clips.

**Surgery for GCaMP imaging**

Emx IRES-cre mice were crossed with Chrm2tdT-D knock-in (BG6.Cg-Chrm2tm1.Hze)/J (Chrm2tdT) mice. Expression of Cre in Emx1-expressing cells allowed for targeting of AAV.hSyn.Flex.GCaMP6f to pyramidal cells, and Chrm2tdT labeled M2 expression, enabling visualization of M2+ patches and M2- interpatches. After anesthetizing, the scalp was retracted, and transcranial imaging of Chmr2tdT expression was performed with a stereomicroscope (Leica MZ16F) equipped with fluorescence optics (excitation 560 nm, emission 620 nm) to determine the location of V1. V1 was visible through the skull as a triangular region of high Chmr2tdT
expression\textsuperscript{55}. Fluorescent images and brightfield images of blood vessels were acquired and overlaid to guide further surgical procedures. A craniotomy was made over the center of V1 in the left hemisphere with a dental drill. First, the skull was thinned in a 3mm-diameter circle where the window was to be placed in the center of V1. Next, a circular flap of skull within this area was removed, exposing the surface of the brain. Four injections (46nl each, 0.3-0.5mm below surface) were performed at different coordinates within V1 (in mm anterior to transverse sinus/lateral to midline: 1.0-1.9/2.4-3.3). Transparent silicone adhesive (Kwik-sil) was applied to the surface of the exposed brain. A 3mm-diameter circular cover slip was placed on top of the Kwik-sil, with the edges of the cover slip contacting the skull at the edge of the craniotomy. A layer of dental cement was then applied to the rim of the cover slip and surrounding bone, rigidly securing the cover slip to the skull. An aluminum head plate containing attachments for head fixation during live imaging was affixed to the skull with dental cement.

2-photon imaging

After surgery, 3 weeks were elapsed to allow for viral expression. Mice were then habituated to the recording apparatus by head-fixing them on a cylindrical wheel on which they could free run (Figure 3.1). Two 30-minute head-fixed habituation sessions were performed in the two days before recording. During each recording session, GCaMP6f signals from L2/3 Emx1-expressing neurons were acquired. An Ultima 2-photon recording system (Prairie Technologies) and Olympus BX61W1 microscope were used with a 20x objective (Olympus UMPanFL, NA 0.46). A mode-locked laser (Mai Tai DeepSee Ti: Sapphire, Spectra-Physics) was used for 920um excitation and GCaMP6f signals were acquired through a green filter (525/70nm). One plane was acquired each session at a sample rate of 3Hz, with a field of view of 0.5 x 0.5mm. Velocity of
the running wheel was collected during the entire recording session and digitized at 10kHz, to determine the onset/offset and free-running speed of the mouse during recordings.

Analysis of GCaMP6f recordings of neuronal activity were performed using Suite2p analysis software\textsuperscript{56} and custom programs in MATLAB. First, registration of images in the time-series was performed to eliminate movement artifacts in the XY plane. Candidate cells were then automatically extracted based on local temporal pixel intensity correlations. For analysis, cells were manually selected based on maximum and mean fluorescence. For each selected cell, fluorescence intensity of the neuropil in a 20µm annulus surrounding the cell body was subtracted from the fluorescence intensity of the cell to determine a value for F. \( \Delta F/F \) was then calculated using a 30-second sliding time window.

\textit{Visual stimuli}

Stimuli were presented on a \( \gamma \)-corrected computer monitor (AOC 27B1H, 34 x 61 cm, 60 Hz refresh rate, 32.1 cd/m\textsuperscript{2} mean luminance) which was placed 33 cm away from the right eye at a 45\(^\circ\) angle to the body axis, subtending 82\(^\circ\) horizontally of visual space. The screen center was elevated to be level with the eye. The Psychophysics Toolbox for MATLAB was used for the creation and presentation of stimuli\textsuperscript{57}. All stimuli consisted of circular square-wave drifting gratings at 100\% contrast which were warped to simulate presentation on a sphere with its center at the mouse’s eye\textsuperscript{58}. At the beginning of each session, receptive field mapping was performed by sequentially presenting a 20\(^\circ\) diameter square wave grating at randomly selected locations in a 4x4 grid. The grid was centered on the visual field of the right eye and grid locations were spaced 15\(^\circ\) apart center-to-center. For each recorded cell, responses to each trial were computed
as the mean ΔF/F during the 4 seconds of stimulus presentation. A 2-dimensional Gaussian curve was then fit to the response profile of each cell (using the MATLAB ‘polyfit’ function), which was used to compute the center of its receptive field. The average X and Y coordinate of the receptive field centers of all responsive cells was computed, and this location was used for centering all stimuli in the remainder of the recording session. The stimulus screen was then turned off, and 10 minutes of spontaneous activity was recorded.

Next, the spatiotemporal tuning of cells was determined by presenting 100% contrast square wave gratings with a range of spatial frequencies, temporal frequencies, and orientations. Peak luminance of light stripes was 62.2 cd/m² and minimum luminance of dark stripes was 2.3 cd/m². A fixed location for these stimuli was chosen to match the receptive fields computed in the first stimulus block. 8 spatial frequencies ranging from 0.01-1.6 cycles per degree, 7 temporal frequencies ranging from 0.1-13Hz, and 7 equally spaced orientations (51° increments) were presented, with 10 repetitions per unique stimulus. When spatial frequency and temporal frequency were not being tested, they were fixed at 0.03 cycles per degree and 2Hz (drift speed = 67°/sec). To determine the preferred spatial and temporal frequency and orientation of each cell, a log Gaussian function was fit to spatial and temporal frequency responses and a von Mises function was fit to orientation responses. A locomotion modulation index (LMI) was computed to determine each cell’s response gain due to locomotion. To compute the cell’s LMI, trials were first classified as locomotion trials if mean forward locomotion during stimulus presentation was >0.1cm/sec, or stationary trials if mean forward locomotion was <0.1cm/sec. The locomotion modulation index was then computed as (R_L – R_S)/(R_L + R_S), where Response during
Locomotion $R_L = \text{mean } \Delta F/F$ on locomotion trials, Response while Stationary $R_S = \text{mean } \Delta F/F$ on stationary trials.

**Aligning recorded cells with $M2^+$ patches and $M2^-$ interpatches**

After completing up to four imaging sessions over four days (1 session/day, using non-overlapping regions of V1), mice were perfused through the heart with 1% PFA. Cortex was flatmounted, postfixed in 4% PFA, cryoprotected in 30% sucrose, and sectioned horizontally at 40µm on a freezing microtome. Images of the cortical window that were previously acquired in vivo were used to locate the recorded regions in ex vivo sections (Figure 3.1A). Sections were imaged at 2X-20X magnification with an epifluorescence microscope (Nikon 80i) equipped with GFP (excitation 490 nm, emission 510-538 nm) and tdTomato (excitation 550 nm, emission 570-720 nm) optics, to determine the $M2^+$ patch pattern of recorded regions. To determine $M2^+$ patches from Chmr2tdT fluorescence, images were first spatially normalized by dividing the intensity of each pixel by the average intensity from a circle with 100µm radius surrounding it\textsuperscript{15}. Images were then blurred with a circular averaging filter of 30µm radius. The image was then divided into six quantiles based on the resulting pixel intensities, with the top three quantiles considered to be $M2^+$ patches and the bottom three considered to be $M2^-$ interpatches. Automated determination of quantile boundaries was determined with custom MATLAB scripts.

Landmarks from the in vivo recording sessions, including blood vessels and GFP fluorescence, were identified in the ex vivo section (Figure 3.1C-D). The ex vivo and in vivo images were aligned based on these landmarks. To account for tissue distortion occurring between recording and sectioning, fiducial points were assigned in in vivo and ex vivo images, which were used for
alignment. Warping was performed using a projective transformation via the MATLAB ‘fitgeotrans’ function. Recorded cells were assigned as M2+ patch or M2- interpatch cells depending on which quantile they aligned with (Figure 3.1H).

**Correlation Analysis**

Activity correlations in darkness (Figure 3.3A) were computed by finding the Pearson correlation coefficient between ΔF/F of each pair of neurons within a session during the 10 minute dark period. To compute stimulus response noise correlations (Figure 3.3B-D), a single response value was first computed for each trial for each cell by taking the average ΔF/F of that cell during the 4 seconds of stimulus exposure. For each unique stimulus condition φ (identical spatial frequency, temporal frequency, and orientation), the Pearson correlation coefficient between the responses of each of a pair of cells across the 10 repetitions of the unique stimulus was computed⁶⁰,⁶¹. The mean correlation coefficient over all unique stimulus parameters (22 unique stimuli from 8 spatial frequencies, 7 temporal frequencies, and 7 orientations) was then used as the overall response noise correlation for the cell pair. Thus each cell pair $i,j$ was assigned a noise correlation value $\rho_{i,j}$

$$\rho_{i,j} = \frac{\text{Cov}[i, j | \varphi]}{\sqrt{(\text{Var}[i | \varphi] \text{Var}[j | \varphi])}}$$

where $i$ and $j$ are the vectors of response magnitudes from cells $i,j$ to each unique stimulus $\varphi$.

To determine whether cells were locomotion-responsive, the Pearson correlation between the ΔF/F trace of the cell and the locomotion velocity during the 10 minute dark period was
computed. Cells were considered significantly locomotion responsive (Figure 3.3C,D) if the p-value of this correlation was <0.05.

**Immunostaining**

To visualize expression of M2 in SST-Cre x Ai9 mice, immunostaining was performed on sections cut on a freezing microtome at 40µm using an antibody against type 2 muscarinic acetylcholine receptor 2. First, sections were preincubated in a blocking solution (0.1% TritonX-100, 10% normal goat serum, PB). Next, incubation was performed in primary rat anti-M2 antibody (1:500, MAB367 [Millipore], 48h at 4ºC) and reacted with Alexa-647-labeled goat anti-rat secondary antibody (1:500, A21247, Invitrogen). Epifluoroscence microscopy was then used to compare M2 expression with SSTtdT expression.

**Anterograde viral tracing**

To anterogradely label projections from MOs and the dLGN, injections of AAV.2/1.hSynapsin.GFP.WPRE.bGH (Penn Vector Core, 46 nl) were performed. Injection targets were (in mm posterior of bregma/lateral of midline/below pial surface): MOs (0.5, 0.5, 0.3/0.5), dLGN (2.35, 2.15, 2.7). For experiments testing the distribution of SST (Figure 3.4), dLGN injections were performed in SST-Cre x AI9 mice. MOs injections were performed in Chrm2tdT mice (Figure 3.6).

**Retrograde viral tracing**

We examined the distribution of V1 neurons projecting to MOs relative to M2+ patches and M2-interpatches with retrograde viral tracer rAAV2-Retro/CAG-Cre (University of North Carolina,
Vector Core) in Ai9 mice. We performed an injection (46nl) in MOp (0.8 posterior of Bregma, 1.25 lateral of midline). To label dLGN terminals in V1, which overlap in L1 with M2+ patches\textsuperscript{12,15}, an injection of anterograde tracer AAV2/1-hSyn-GFP was in addition made into dLGN. Three weeks after injection, mice were perfused with 1% PFA, the cortex was flatmounted, postfixed with 4% PFA, cryoprotected in 30% sucrose, and cut on a freezing microtome at 40µm in the tangential plane. Immunostaining against M2 was performed in tangential sections to delineate areal borders (Figure 3.6H). Sections were wet-mounted on slides and images were acquired with epifluorescence microscopy at 4-40X magnification, which were used to delineate M2+ patch borders and identify labeled cell bodies and dendrites. The remainder of the brain was separated from the cortex, sectioned at 40µm in the coronal plane, and epifluorescence images were obtained to confirm the location of dLGN injection.

**Quantification of projection strength**

To determine the strength of anterogradely labeled axonal projections or SST expression in M2 intensity quantiles, M2 expression images were high pass filtered and blurred. M2 images were then divided into 6 intensity quantiles. The average optical density from projection or SST\textsubscript{tdT} images within each quantile region was then found. The mean and standard error of intensity within each quantile was computed across multiple subjects and plotted (Figures 4G, 5E). Pearson correlation coefficient and p-value between quantile and intensity was computed, to determine whether projections or SST expression were associated with M2+ patches or M2-interpatches. SST\textsubscript{tdT} expression distribution was also compared against a shuffled distribution. SST\textsubscript{tdT} images were downsampled by averaging all pixel intensities within a 5x5µm square, the locations of these units were shuffled, then mean intensity within each M2 quantile was
measured, using the original M2 quantile borders. Analysis of the correlation between quantile and shuffled intensity was performed, as with the non-shuffled images (Figure 3.4H).

Quantification of dendrites in M2+ patches and M2- interpatches

Images of dLGN inputs to L1 were acquired as a proxy for patchy M2 expression\textsuperscript{12,15}. High-pass filtered and blurred images were divided into six intensity quantiles and the top three quantiles were treated as M2+ patches while the bottom three quantiles were treated as M2- interpatches. Retrogradely labeled apical dendrites of MOp-projecting cells were identified by morphology (tapered and accompanied by dendritic spines) and by diameter (0.4-1.5µm)\textsuperscript{62}. Dendrites were manually traced from 40X images of tdTomato expression and the total length of dendrites in M2+ patches and M2- interpatches was computed. A permutation test was then performed by shuffling the locations of labeled dendrite lengths and determining the resulting length ratios in M2+ patches and M2- interpatches, while maintaining the original patch/interpatch borders. The original length ratio was considered significantly different from 1:1 if it fell outside of the 95% bounds of the distribution of ratios generated by $10^6$ shuffling iterations. Custom MATLAB scripts were used to perform these analyses.

3.6 REFERENCES


Chapter 4: Conclusions and Future Directions

In this thesis I performed two studies aimed at addressing how top-down, behaviorally relevant feedback interacts with M2 modules in mouse visual cortex. In the first study (Chapter 2), I used pathway tracing to determine the connectivity of the postrhinal visual area (POR) with brain structures involved in navigation and emotional behavior, including the amygdala, medial entorhinal cortex, and the lateral posterior nucleus of the thalamus. I found that M2+ patches preferentially receive inputs from visual nuclei in the thalamus, while M2- interpatches preferentially receive inputs from the amygdala, a central regulator of affective behavior. Retrograde tracing revealed that M2- interpatches provide the majority of POR outputs to the amygdala and medial entorhinal cortex, an area important for detecting spatial landmarks and determining the location of the animal in its environment. This study demonstrated that M2 modules in higher visual cortex have distinct connection profiles with structures regulating behavior, and that M2- interpatches may play an important role in associating emotional valences with landmarks and spatial locations.

In the second study (Chapter 3), I used calcium imaging and visual stimulation in awake, behaving mice to determine how cells in V1 M2+ patches and M2- interpatches change their response and network activity in the context of two behavioral states, locomotion and rest. I found that M2- interpatch cells show a greater increase in visual responses during locomotion, while M2+ patch cells are more selectively tuned for stimulus orientation. Comparison of correlated stimulus responses showed that M2- interpatch cell pairs have increased noise...
correlations between cells spaced at long distances apart, compared to M2+ patch cell pairs. I found two anatomical features which may underlie this increased M2- interpatch locomotion modulation: preferential feedback from secondary motor cortex and inputs from inhibitory somatostatin-positive interneurons. Finally, retrograde tracing demonstrated that L5 projections neurons and apical dendrites aligned with M2- interpatches provide outputs to the primary motor cortex, suggesting that M2- interpatch cells contribute to the guidance of motor behavior.

4.1 M2 MODULARITY IN V1 AND HIGHER VISUAL AREAS

The results summarized above raise the question of whether there is a general principle of M2 modularity that characterizes the roles of M2+ patches and M2- interpatches in both V1 and higher visual cortex. A commonality that emerges from both of these studies is that M2-interpatches appear to be more connected with and modulated by higher-order areas directly responsible for driving behavior. In POR, these include the amygdala and ENTm, while in V1 they include PM1, MOp, and MOs (Figures 3.5, 3.6). Physiologically, this greater degree of modulation by behavior is demonstrated by increased locomotion modulation of M2- interpatch cells (Figure 3.2). Locomotion may also transiently enhance bidirectional communication between V1 M2- interpatch cells and areas controlling behavior, as a recent study found that activity correlations between V1 cells and MOs are increased during locomotion2.

Another general principle of M2- interpatches may be that they receive L1 inputs which are less retinotopically specific. Amygdala projections to cortex, which favor M2- interpatches in POR, have been shown to be non-topographic, in comparison to the strict retinotopy of dLGN and LP inputs targeting M2+ patches in POR (Figure 2.5). Within V1, inputs driving locomotion
modulation (including possibly those from MOs) are not retinotopically specific. The visual thalamic inputs targeting V1 M2- interpatches from LP are less retinotopically precise than those targeting V1 M2+ patches from the dLGN shell. These properties suggest that M2- interpatch cells detect wider-field stimuli than M2+ patch cells. In V1, these wide-field stimuli may include elongated edges or optic flow, while relevant wide-field stimuli in POR may include entire visuospatial contexts which can be associated with emotional valence.

A general feature of M2+ patches is that they receive L1 inputs from areas more specialized for visual processing than for multimodal integration, including LM, AL, dLGN, and LP in the case of POR inputs (Figure 2.5). This feedback to M2+ patches may tune responses based on visual context rather than behavioral context, for example through surround suppression, which is driven by LM→V1 feedback. A recent study on the function of extrastriate areas during visual discrimination and detection tasks illustrates this proposed division in the case of areas providing V1 inputs. It was shown that optogenetically disrupting LM and AL, which target M2+ patches in V1, impaired orientation discrimination. Disrupting PM, which targets M2-interpatches in V1, interfered with detection task performance without regard to sensory features of the stimulus. PM played a retinotopically nonspecific role in this detection task, in accordance with the retinotopically nonspecific nature of behaviorally relevant feedback to M2- interpatches.

In both V1 and extrastriate areas, M2+ patches have not been shown to preferentially provide outputs to downstream targets directly involved in driving behavior, as M2- interpatch cells have. However, M2+ patches may be involved in visual processing during states where no urgent behavioral objective, such as fleeing, fighting, or hunting, is being pursued. These states
are likely to not involve locomotion, which is in accord with the lower locomotion modulation of
M2+ patch cells (Figure 3.2), as well as their tuning for shape rather than for motion. Unlike M2-
interpatches, M2+ patch cells also appear to be specialized for discriminating objects occupying
a limited portion of the visual field. This is suggested by the more retinotopically precise nature
of inputs to M2+ patches in L1, reduced long-range noise correlations between M2+/M2+ cell
pairs (Figure 3.3B), and lack of intra-areal long-range projections between V1 M2+ patches (Ji
W., D’Souza R.D., Burkhalter A., unpublished data).

LP inputs to L1 present an interesting divergence between higher and lower visual cortex,
targeting M2- interpatches in V11 and M2+ patches in extrastriate areas (Figure 2.5). One
possible explanation for this difference is that targeting of M2+ patches or M2- interpatches
depends on the hierarchical level of the area providing input, relative to the hierarchical levels of
the other sources of input to L1 of the targeted area. The primary sources of input to V1 are
lower-order visual nuclei and areas, among which LP is relatively a higher-order source of input,
according to a recent large scale anatomical study which ranked the hierarchical levels of
thalamic nuclei and cortical areas17. Due to this relatively high hierarchical position, LP targets
M2- interpatches in L1 of V1. Conversely, L1 of extrastriate areas, including POR, receive inputs
from visual areas, multimodal areas, and the amygdala, which may be considered a higher-order
source of feedback due to its connectivity with frontal areas18–20, predominant projections to L1
and deep layers of multimodal association areas21–23 (Figure 2.5F), and its lack of connections
with V121. Within this group of input sources to extrastriate cortex, the LP has a relatively low
hierarchical level, and thus provides inputs to M2+ patches, while amygdala inputs target M2-
interpatches.
The presence of M2 modules in mouse visual cortex introduces a larger question: is there a functional advantage to organizing cortical areas into interspersed modules containing two different cell types, rather than separating these cell populations into completely separate areas? Based on the prominent differences in feedback to M2+ patches and M2- interpatches, I suggest that one advantage of interspersing these modules is that it allows for two types of top-down modulation within a single area. The connectivity of M2- interpatches enables spatially nonspecific feedback modulation to adapt visual responses based on behavioral context and goals. The presence of M2+ patches with retinotopically specific feedback modulation enables top-down feedback to enhance or tune the responses of cells in a particular part of the visual field, for example, to focus attention on a particular, small object. Thus the presence of M2 modularity throughout the mouse visual cortex hierarchy suggests that both types of contextual feedback are essential components of visual processing in areas with diverse functional roles.

4.2 LOCOMOTION AND NOISE CORRELATIONS IN M2- INTERPATCH CELLS

Previous research has shown that locomotion, like attention in studies involving primates, causes noise correlations to be reduced in locomotion-modulated cells in V1, improving stimulus discriminability. This phenomenon may appear to contradict the finding in Chapter 3 that noise correlations were increased in a population of M2- interpatch cells which were highly locomotion-modulated (Figure 3.3B, C). An important feature of correlations between these M2-interpatch cells, however, is that increased noise correlations (relative to M2+ patch pairs) were specifically found among M2- interpatch cell pairs that were spaced at long distances apart, with correlations increasing along with distance when spacing was greater than 250µm. Previous
studies demonstrating decorrelation during locomotion in mouse have in almost all cases recorded cells aligned in a single cortical column and thereby representing the same retinotopic location\textsuperscript{25–27}. Conversely, simultaneous wide-field calcium imaging and multi-unit activity recordings have demonstrated that locomotion increases the correlated activity of single V1 neurons with the average activity across V1\textsuperscript{2}. The distantly spaced M2- interpatch cell pairs with increased noise correlations which I recorded may therefore exhibit different population responses than pairs which are closely spaced and which become less correlated during locomotion. The reason for this difference may be that reducing noise correlations only increases information about the stimulus in spike counts if the decorrelation occurs in neurons tuned to respond to similar stimuli\textsuperscript{28,29}. M2- interpatch cell pairs which are hundreds of microns apart, rather than in the same cortical column, may represent different stimuli, in which case increased noise correlations would not reduce stimulus information represented in the responses of these cells.

4.3 FUTURE DIRECTIONS
An important remaining question about M2 modularity is the relationship between the location of pyramidal cell somas and their apical dendrites in L1 with respect to M2+ patches and M2-interpatches. D’Souza et al. (2019)\textsuperscript{1} investigated this question with regard to parvalbumin-positive interneurons (PV cells). However, it is unknown whether pyramidal cells receive apical dendritic input from the same L1 module with which they are aligned. The previously used methodology of biocytin-filling individually patched cells is reliable but low throughput, considering the diversity of dendritic morphology in mouse V1\textsuperscript{30}. An alternative would be to use the Brainbow 3.0 system\textsuperscript{31} to simultaneously express multiple colors of fluorophores in many
pyramidal neurons and their dendritic trees, by crossing transgenic Brainbow mice with a mouse expressing Cre in Emx1-positive (pyramidal) neurons. The cortex could then be removed, flattened, and immunostained for M2 with an Alexa 647-tagged IgG, which has minimal spectral overlap with the Brainbow fluorophores. Visual cortex fluorescence would then be imaged with confocal microscopy and dendritic trees of individually labeled pyramidal cells reconstructed. The location of each cell’s soma and dendritic branching in L1 could then be compared to M2+ patches. This would reveal whether somas aligned with a given M2 module receive L1 input from that same module, or whether there is a more complex relationship between soma and dendritic tree alignment.

One essential improvement that could be made to the methodology I used in Chapter 3 would be using in vivo imaging of M2 expression, rather than relying on imaging of M2 expression in ex vivo sections. In the experiments described, the presence of blood vessels and the low fluorescence intensity of Chrm2-tdTomato made determination of M2+ patches unreliable in vivo, making it necessary to determine the location of M2+ patches ex vivo and align these with in vivo recorded images (Figure 3.1). While alignment was usually successful and only a minority of cases had to be discarded due to poor alignment, this process added a possible margin of error in assignment of cells to M2+ patches or M2- interpatches. Reliable in vivo imaging of M2+ patches would eliminate the possibility of alignment error and greatly reduce processing time, as ex vivo processing would no longer be necessary. Cells in deeper layers (L4 or L5) could be imaged and reliably aligned at the time of recording with M2+ patches, whereas performing this alignment ex vivo introduces an additional possibility of error due to the greater number of sections separating L1 M2+ patches and the deeper layer cells. This improvement
would also eliminate the need for future experimenters investigating M2+ patches in vivo to perform ex vivo flatmounting and tangential sectioning of mouse cortex, which are not widely used techniques. Reliable in vivo imaging of M2+ patches might be accomplished by using a brighter fluorophore or optimizing the 2-photon recording conditions to avoid the optical distortion of tdTomato fluorescence introduced by blood vessels. Alternatively, injections of anterograde tracer into the dLGN, which have proven to be effective at clearly labeling M2+ patches, could be performed prior to recording as a means of labeling M2+ patches for viewing in vivo. One limitation of this strategy would be that damage to the dLGN from the injection might disrupt responses in visual cortex, which receives dLGN input.

Further extension of the research described here could also include studying the activity of M2+ patch and M2- interpatch cells in higher visual cortex during complex behavior. Experiments may involve recording the activity of these cells with calcium imaging in the context of reward, navigation, or both. For example, head-fixed mice could be trained to navigate a maze in virtual reality for a food reward while activity of cells in POR M2+ patches and M2- interpatches is recorded with 2-photon microscopy. A hypothesis that could be tested is that M2- interpatch cells and not M2+ patch cells become responsive to the visual stimuli and locations within the virtual maze associated with food reward. Meanwhile, M2+ patch cells would be predicted to develop place fields and be tuned for visual features presented on the screen, such as edges, but not have activity modulations in response to behavioral variables, such as locomotion or reward. This or similar experiments would provide greater insight into the role of modular organization in the visual guidance of complex, goal-directed behavior.
4.4 REFERENCES


12. Jin, M. & Glickfeld, L. L. (2020). Mouse higher visual areas provide both distributed and specialized contributions to visually guided behaviors. Current Biology 30: 1–11


