Investigating the Anatomical Basis for Streams in the Mouse Visual Cortex

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ABSTRACT

Examining the organization of the visual cortex can help show how visual processing is accomplished. For instance, a major organizational property of the primate visual cortex is the division of the higher visual areas (HVAs) into separate streams with high connectivity and similar functional properties. These streams allow for parallel processing of functionally distinct visual phenomena, with the ventral stream focusing on object recognition and the dorsal stream on localization and movement coordination. While some mechanisms for the segregation of streams from primary visual cortex (V1) to the HVAs are known, such as the clustering of neurons feeding into separate streams within specialized layers, the architecture at the level of single cells is not known. Understanding this anatomical organization is important for predicting how information is distributed, and shared, among the HVAs. In the mouse, studies examining the reciprocal connectivity between visual areas, especially Burkhalter (Wang, Sporns, & Burkhalter, 2012), have suggested a stream organization similar to that seen in primates. Modern genetic techniques in the mouse present an opportunity to study the anatomical organization and functional specializations that contribute to parallel processing in streams. Towards this goal, we set out to understand which neuronal populations in mouse V1 might participate in each stream and their degree of anatomical segregation.

Here we tested the hypothesis that there is anatomical specificity, at the level of both laminar populations and individual neurons, in the makeup of streams originating in V1. Anatomical specificity was assessed by quantifying the number of neurons in each V1 lamina projecting to each HVA and by finding the probability that V1 neurons project to multiple HVAs. Although laminar specificity did not differ among the HVAs, there was a trend of V1 neurons with multiple projection targets synapsing onto HVAs in a preferred stream. Though
these findings do not reveal the anatomical basis for streams seen in the primate cortex, they reveal a mechanism for sharing information among streams that could be crucial to understanding these modules’ role in visual processing. Future studies could investigate the functional properties of neurons with multiple projections to HVAs in a certain visual stream and determine physiological aspects that could contribute to sharing of information among streams with different response properties. These studies will ultimately reveal how the visual system distributes information into streams made up of highly interconnected HVAs, and how these networks are used to guide behavior.

**BACKGROUND AND SIGNIFICANCE**

The nuances of structure can tell much about how an organism experiences and reacts to the world. This relationship between form and function has guided multiple significant advances in biology, with the most famous example being Charles Darwin’s observations of variations in beak shapes contributing to his theory of natural selection (Darwin, 1859). In neuroscience, anatomical studies have been central to our understanding of the brain. Ramon y Cajal’s staining of individual neurons and observation of their structure allowed him to validate neuron doctrine and thus plant the beginnings of modern neuroscience (Cajal, 1889). Study of the visual systems has especially benefitted from examining how the anatomical organization of the brain might contribute to processing. Hubel and Wiesel present the most classic example of this line of thought in their discovery of organizational columns in the cat visual cortex (1962). These anatomical studies laid the groundwork and provided rationale for future studies investigating the hypothesized functions of these structural features. In modern neuroscience it is still crucial to elucidate the anatomical form of a brain network before diving into its functional mechanisms.
Today’s studies especially have to account for the nuanced anatomical and functional differences across species in order to take full advantage of the wide array of genetic tools available for approaching the brain at all levels of inquiry. Additionally, comparing anatomy across species can distinguish neurological features that are fundamental versus those that are species specializations. A variety of organisms have been used to study the mechanisms of the human brain. These animals encompass a wide range of species, including primates, cats, flies, leeches, and mice (Theys, Romero, van Loon, & Janssen, 2015; Briggman, Abarbanel, & Kristan Jr, 2005; Niell & Stryker, 2008). As our understanding of these animals’ neural systems has progressed, we have uncovered key differences between species and how their neural anatomical makeup and physiological mechanisms might differ from those of humans. For instance, rodents do not display the organizational columns in the visual cortex that are seen in cats and primates (Ohki, Chung, Ch’ng, Kara, & Reid, 2005). Neuroscience research then must find fundamental features of structures involved in visual perception that persist across species despite fluctuations in specializations such as cortical columns.

Through manipulation of small scale circuit and molecular mechanisms that persist across species, modern genetic methods offer one way to accomplish this inquiry (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005). In the mouse visual system they have already been used extensively to give insights into structural and mechanistic features of visual processing (Glickfeld, Histed, & Maunsell, 2013; Kim, Juavinett, Kyubwa, Jacobs, & Callaway, 2015). Our knowledge of the primate system can guide mouse visual research to help us find more basic features of visual processing relevant to the human brain.
The anatomical basis for streams in the primate visual cortex

The dorsal and ventral streams are widely accepted as major anatomical organizational features of the primate visual cortex. This anatomical segregation reflects two different functional modes of visual processing. Over the years a nuanced distinction has arose that compares the recognition of an object to actions associated with such an object, as opposed to simply localizing it (Goodale & Milner, 1992). Anatomically, this theory distinguishes projections from the striate to the inferotemporal cortex versus those from the striate to the posterior parietal region, termed the ventral and dorsal streams, respectively. Visual processing is aided by the distinct functions of these two streams, with the ventral stream responsible for recognizing objects while the dorsal stream aids in guiding motions respective to these objects.

Anatomical segregation enables parallel input in earlier visual processing that separates information in preparation for the stream organization in the HVAs (Figure 1). For instance, layer 4B of V1 receives input indirectly largely from magnocellular pathways of the retina. In a similar fashion, layer 2/3 receives information from parvocellular retinal pathways (Fitzpatrick, Lund, & Blasdel, 1985). These layers also share the response properties of these pathways, with 4B sensitive to movement and 2/3 to color and shape (Hawken, Parker, & Lund, 1988; Livingstone & Hubel, 1984). Though more contemporary research has showed this organization is more complex (Callaway & Wiser, 1996), the retinal pathways are largely conserved in these V1 layers.

This segregation continues in V1 projections to the secondary visual area (V2). V2 is made up of pale stripes, thick stripes, and thin stripes, with the thick and pale stripes being differentiated by a cytochrome oxidase stain. Classically, layer 4B projects largely to the thick stripes of V2 with layer 2/3 projecting to the pale and the thin stripes (Livingstone & Hubel,
This allows for another level of processing while still maintaining the two parallel pathways.

Figure 1: Layer specificity in the primate visual cortex. Parvocellular and magnocellular inputs originating in the retina are largely segregated into layer 2/3 and layer 4B of V1, respectively. This separation is continued in V1 inputs to V2 as layer 2/3 projects to the pale and the thin stripes while layer 4B projects to the thick stripes. Finally, this information is separated into streams of the higher visual areas as V4 receives inputs from the pale and thin stripes while MT has neurons projecting to it from the thick stripes. In each of these areas a functional segregation is maintained as well, with areas with a parvocellular source responding to form and color and those of the magnocellular pathway being sensitive to movement.

Layer-specific projections from these visual areas to the initial HVAs of the dorsal and ventral streams then finalize this segregation. The thick stripes of layer V2 project to higher visual area MT, while the thin and pale stripes project to area V4 (Shipp & Zeki, 1985; Shipp & Zeki, 1989). This anatomical specificity is also accompanied by functional segregation, as the thick stripes are sensitive to movement while the pale and thin stripes have response properties corresponding to form and color. Consequently, the primary visual areas have layers with functional properties that correspond to the stream into which they feed, which reflects a segregation of response characteristics that occurs earlier than the extrastriate cortex and is central to visual processing.
Once visual information reaches the HVAs it is then divided into the dorsal and ventral streams, which begin with areas MT and V4, respectively. The functional differences between these two areas reflect the parallel functionality of the two streams. For instance, V4 is specialized for responding to color and shape (Schein & Desimone, 1990; Pausupathy & Connor, 1999). Neurons in V4 exhibit sharp spectral tuning curves, which might imply their response properties pertain to colors. In fact, lesions to area V4 impair the color perception abilities of macaques (Walsh, Carden, Butler, & Kulikowski, 1993). Additionally, V4 cells respond to contour features, such as curves and angles, which would be necessary for identifying the outlines for the shape of an object. These properties contrast the behavior of MT cells, which are highly selective for aspects of motion (Albright, 1984). This selectivity might help explain the deficits in motion perception that are observed after lesions to this area (Newsome & Pare, 1988). Motion perception is necessary for guiding actions to objects in space. Consequently, the structural organization of streams is reflected in the functional dichotomy of the areas in these streams.

Still, there are more complex aspects of visual processing, such as face recognition and spatial localization, that we have not fully elucidated. Understanding the organization of information in the dorsal and ventral streams can help in explaining these features of visual processing. Finding anatomical structures central to the organization of streams can inform hypotheses relating to functional mechanisms of parallel processing and how it streamlines aspects of visual information for use by other parts of the brain. A variety of methods, including some whose origins date back to the late 19th century, provide tools for dissecting this anatomical organization.
Early anatomical neural methods

Some of the first investigations into neural anatomical organization focused on localizing functions to certain areas of the brain. In the first half of the 19th century, phrenology became one of the first sciences to posit localization of brain function (Greenblatt, 1995). It did this by assuming brain function and behavioral abilities based on differences in skull shapes, which was thought to reflect differences in brain structures. Although it was quickly discredited and is now considered a pseudoscience, phrenology was influential in its idea that different parts of the brain are specialized for different functions.

Tracing techniques provide a legitimate and accurate way of localizing function and studying anatomical features that can help visualize structural details. The first useful method of staining was accomplished by Camillo Golgi, who discovered a way to fill nerve cells in black using a combination of potassium dichromate and silver nitrate (Golgi, 1873). This technique came to be known as Golgi staining. The main advantage of it is that by some chemical phenomenon only a fraction of the neurons exposed to the stain are actually filled. As a result it is possible to view the detailed features of individual neurons, which Ramon y Cajal would take advantage of when making his neuron drawings (Cajal, 1889). Golgi’s methodology would go on to inspire tracing studies examining the visual system. For instance, deprivation studies examining development in inputs to the visual cortex used reduced-silver stain to show shrinkage in visual cortical columns corresponding to the sutured eye (Hubel, Wiesel, LeVay, 1977). This helped reveal the structure of the visual cortex, how it develops, and the connectivity from the eye to the higher processing areas. Still, tracing using this method is largely unspecific for neuron type and projection target. Our understanding of the visual system largely depends on assigning functions to different areas. Consequently, a method that labels neurons projecting to a
specific area would show the anatomy but also tell something of the functionality of these neurons. With older staining methods that label indiscriminately then, it is more difficult to tell the functional significance of the visualized path. More specific tracing allows for analysis of detailed structures, such as subtypes of neurons specific to one V1 layer or cells synapsing onto a certain HVA. Structures such as these would help form an anatomical basis for streams.

*Modern genetic anatomical methods*

Modern genetic methods avoid many of the challenges posed by classic techniques and allow for investigation of precise organizational anatomical features. The Cre recombinase and lox p system has proven useful for tracing neuronal projections (Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007). This technique takes advantage of a mechanism present in some bacteriophages to cause specific expression of fluorescent molecule in the desired set of neurons (Lobocka et al., 2004). Cre recombinase accomplishes this by excising or flipping the transgenic mouse genome in between the lox p sites in order to eliminate a stop codon and cause transcription of a fluorescent protein gene. As a result, injection of an adeno-associated virus containing Cre recombinase can cause translation of fluorescence in specific types of cells or locations in the brain if a gene for a fluorescent protein, such as td Tomato, is in between the lox p sites (Ahmed et al., 2004). Since td Tomato can be expressed exclusively in pyramidal cells, this is convenient for elucidating structural architecture made up of excitatory neurons specifically. Additionally this feature can label neurons projecting to or from a specific brain area.

Whether neurons afferent or efferent to the area of interest are labeled depends on whether the injected virus travels anterograde or retrograde. In this way, genetic methods for anatomical labeling can be fit to adapt to a variety of experimental approaches. For instance, the virus that causes expression of Cre recombinase can travel retrogradely and infect the neurons
that are projecting to the area injected (Rothermel, Brunert, Zabawa, Diaz-Quesada, & Wachowiak, 2013). This is helpful if a gene for a fluorescent molecule like tdTomato is flanked by lox p sites in the presynaptic neuron, in which case all of the neurons projecting to the injected site will be labeled with tdTomato. Similarly, viral vectors expressing GFP can be useful in anterograde labeling of a neuronal population (Oh et al., 2014). In combination then, these viruses can mark an area of interest and then label neurons in that area that are projecting to a specific location in the brain.

This specificity makes viral techniques more valuable than classic methods. For example, mice can be bred so that Cre will only be expressed in a certain cortical layer (Gong et al., 2007). This can be useful in labeling populations of neurons projecting to a certain layer, which might help in elucidating mechanisms involving laminar specificity. Additionally, mice can be generated so that different types of neurons express different fluorescent proteins (Kim et al., 2015). As a result there are a myriad of ways to isolate functionally relevant structures and cell types in the brain in order to truly dissect their anatomical organization when studying pathways of visual processing.

*The organization of the mouse visual cortex*

The organization of the mouse visual cortex and its relation to that of the primate cortex has become increasingly relevant with these new genetic technologies that make the mouse brain a better model for studying visual processing at the circuital, neuronal, and molecular levels. Mouse visual cortex is made up of a primary visual area (V1) with higher visual areas (HVAs) surrounding it at its anteromedial and anterolateral sides. Information enters the cortex at V1 and then is distributed to the surrounding HVAs (Wang & Burkhalter, 2007). Neurons in area V1 are generally responsive to lines at a particular orientation, but these neurons are not arranged so that
those that respond to similar orientations are neighboring each other, as is seen in the primate cortex (Ohki et al., 2005). Additionally, the neurons in V1 respond to a varying spatial and temporal frequencies, yet each usually projects onto an HVA with response properties similar to its own (Glickfeld, Andermann, Bonin, & Reid, 2013). Consequently, the HVAs show more functional specialization than what is observed in V1. Three major HVAs are the anterolateral area (AL), the posteromedial area (PM), and the lateromedial area (LM). Generally, LM has similar properties as V1 and is analogous to V2 in primates, while AL responds to objects moving at fast speeds and PM responds to slow-moving stimuli (Andermann, Kerlin, Roumis, Glickfeld, & Reid, 2011). This shows sharp differences in specialization between the HVAs, which might mirror the differences that are seen between the dorsal and ventral streams of the primate visual cortex.

Some level of specialization is also seen within V1. For instance, there are major differences in response properties between the cortical layers of V1. Layer 5 has a lower average orientation selectivity and higher tuning width than the other layers, while layer 2/3 has the lowest average tuning width (Niell & Stryker, 2008). Additionally, layer 6 has the lowest preferred spatial frequency. In some way these differences are parallel to the differences seen between streams and between layers of the primary visual areas in the primate. Recent evidence also shows that mouse V1 neurons may be specialized by cell type, with neurons in layer 5 exhibiting three distinct variants that differ in projection pattern and response properties (Kim et al., 2015). This adds yet another layer of specialization that might add to an anatomical organization allowing for parallel processing in the mouse HVAs.

Since neurons in V1 are not organized structurally by response properties, it would follow that projection patterns are determined by cell types. In fact, although the primate
generally shows layer specific projections (Shipp & Zeki, 1985; Shipp & Zeki, 1989), more contemporary research has shown that cell subtype in each layer more accurately describes the neuronal populations projecting to each HVA (Yabuta, Sawatari, & Callaway, 2001). Consequently there are multiple methods of specializing the projections from V1 to the HVAs, of which laminar specificity is only one.

Overall, these observations imply that the mouse has a similar pair of extrastriate processing streams like what is seen in the primate visual cortex. In fact, lesion studies have shown that areas in the rodent brain corresponding to the locations of the primate dorsal and ventral streams also retain similar functions. For instance, lesions in the parietal cortex impaired ability in spatial tasks (Tees, 1999). In accordance with these findings, ablations in the temporal cortex led to failures in object recognition (Ho et al., 2011). These functional discoveries are reinforced by anatomical connectivity between the mouse HVAs. By examining the interconnectedness of the HVAs it was hypothesized that a ventral and dorsal module exist in the mouse extrastriate cortex, with LM belonging to the ventral module and AL and PM belonging to the dorsal one (Wang et al., 2012). These findings offer a strong case for an anatomical stream organization in the mouse.

Experimentation questions, approaches, and significance

Despite evidence supporting stream organization in the mouse visual cortex, it is still not clear how the neuronal populations of V1 segregate information for participation in each stream. Knowledge of this mechanism would help in understanding the functions of these streams. Namely, revealing detailed structural architecture that provides a basis for streams may show how information is dissected into discrete mechanisms for visual processing. Here, I will test the hypothesis that there is anatomical specificity, at the level of both laminar populations and
individual neurons, in the makeup of streams originating in mouse V1. Understanding this anatomical organization is important for predicting how information is distributed and shared among the HVAs.

Two approaches will be used to test for this anatomical specificity. First, I will determine the degree of laminar specialization in projections from V1 to the HVAs. This will be accomplished by identifying the cells in V1 that project to one of three HVAs of interest: LM, AL, or PM. For each HVA, retrograde labeling will be used to find the neurons in V1 that project to it, and the proportion of labeled cells present in each V1 layer will then be calculated.

Previous studies have suggested various stream organizations involving the HVAs LM, AL, and PM (Wang et al., 2012). If these proportions differ significantly between HVAs, then V1 laminar specificity would be supported as a mechanism contributing to the anatomical basis for separate visual streams in the mouse (Figure 2, left). If, however, the proportions do not differ across
HVAs, then there would be a lack of laminar specialization in projections from mouse V1 to the HVAs, which would imply a crucial step in primate development and evolution (Figure 2, right).

Second, I will determine if individual V1 neurons project to multiple HVAs in the same visual stream. This will be accomplished by examining the extent to which V1 neurons projecting to one HVA also project to other HVAs (Figure 3). If V1 neurons do project to multiple extrastriatal targets, I would expect single neurons to send information to HVAs of the same stream. This finding would strongly support an anatomical basis for streams in the mouse visual cortex, especially if accompanied by results suggesting laminar specificity.

**Figure 3:** Possibilities for V1 axonal projection patterns. The middle panel shows a structure where each V1 neuron projects to a single HVA. The right panel depicts a framework where each V1 neuron projects to each HVA. On the left is an anatomical structure where a V1 neuron projects to multiple HVAs in the same stream. This middle configuration would provide the strongest anatomical basis for streams in the mouse cortex.

Future studies could then investigate the functional properties of stream-specific V1 layers and neurons. Past work would predict that neurons share similar functional properties with the stream to which they project (Glickfeld et al., 2013), but possibly there is also a local structure to similar neurons in V1. Ultimately, these studies will reveal how the visual system distributes information into streams made of highly interconnected HVAs, and how these networks are used to guide behavior.
METHODS

Mice

All procedures were conducted in accordance with the ethical guidelines of the NIH and approved by the IACUC at Duke Medical Center. Sixteen mice (both sexes; six flex-tetramino and ten C57 wild type; group housing on a regular light-dark cycle) were used in this study.

Cranial Window Implant and Habituation

Dexamethasone (4mg/ml, IM) was administered at least three hours before surgery. Ketamine (10 mg/ml, IP), xylazine (0.5 mg/ml, IP), and isoflurane (4% in oxygen gas before surgery, 1-2% during surgery) were used to anesthetize animals. Mice were also given meloxicam (0.5 mg/ml, IM) at surgery onset as a prophylactic non-steroidal anti-inflammatory drug to help manage pain. A headpost was attached to the skull using C&B metabond (Parkell), and a circular craniotomy of diameter 5 mm was made over the left visual cortex, with the center being 3.1 mm lateral and 1.64 mm anterior to lambda. This allowed for implantation of a cranial window, which was made up of an 8mm coverslip (Warner) bonded to two 5 mm coverslips. Coverslips were bonded using optical adhesive (Norland no. 71), and the window was attached using C&B metabond (Parkell). Mice were given three injections of buprenex (0.01 mg/ml, IM) spaced twelve hours apart for post surgery analgesia. They were also given three injections of the antibiotic cefazolin (25 mg/ml, IM) also spaced twelve hours apart to prevent infection. Additional injections of cefazolin were given if mice began to show signs of infection. Animals were left to recover for at least one week.

Prior to imaging, mice were habituated to the rig in which they would be held. The rig consists of a platform, a free-spinning wheel, and two head clamps. Rigs were assembled with parts from ThorLabs. During imaging mice are held clamped in place while their feet are free to
move on the wheel. Mice were habituated for increasing durations until they could walk on the wheel without appearing distressed and uncomfortable. This was usually accomplished after three sessions of 20 minutes, 40 minutes, and 60 minutes.

*Widefield Imaging*

Epifluorescence imaging was used to measure changes in intrinsic autofluorescence during visual stimulation in order to map V1 and the HVAs (Husson, Mallik, Zhang, & Issa, 2007). Visual stimulation consisted of circular visual gratings on a monitor 21 cm from the mouse’s right eye. All drifting gratings were at a temporal frequency of 2 cycles per second and a spatial frequency of 0.1 cycles per degree. Visual gratings appeared at four locations at either 10° or 0° elevation and 50° or 65° from the azimuth, with the azimuth being defined as the mouse’s sagittal plane. During visual stimulation, autofluorescence was excited by blue light (470 nm wavelength) and measured using a green and red emission filter (long pass, 500nm cutoff). A CCD camera (QImaging) was used to collect images through a 5x air immersion objective (Mitutoyo). Micro-Manager 1.4 was used to acquire images (exposure time was 500 ms with a frame rate of 2 frames per second) and trigger presentation of visual stimuli (on for 10s, interleaved with 10s of mean luminance). Using ImageJ software the fluorescence was averaged across frames for each position, which yielded four images, each one showing activation corresponding to the location of visual stimulation presented. The images with the most robust outlines of fluorescence were combined to give a retinotopic map showing the borders of the visual cortical areas of interest (Figure 4).
Targeted Injection of Viruses

These retinotopic maps were used for targeted viral injections into V1 and the HVAs for expression of fluorescent proteins. Dexamethasone was administered at least 3h before window removal. Mice were anesthetized (isoflurane, 1-1.5%) and the cranial window was sterilized with alcohol to prepare for removal. A volume injection system (30 nl/min) was then used to inject the desired viruses in an ACSF solvent (Figure 5). Injections were conducted in two parts, with half of the volume being injected at 250 microns below the pia and the other half at 500 microns below. The vasculature was used in combination with the retinotopic maps to guide injections the HVA of interest (either AL, LM, or PM) and to V1. A new cranial window was then implanted made of one 8 mm coverslip and three 5 mm coverslips.
Figure 5: Table of the viruses used for injections. Cre was generally injected into an HVA at a lower injection volume while Egfp and flex.tdTomato were injected into V1 to cause anterograde labeling in large volumes.

After injection, the mice were returned to the colony for two weeks to give time for expression of virus. Wide-field epifluorescence microscopy was used to confirm expression of fluorescence in the correct locations (Figure 6). Mice lacking fluorescence or with fluorescence in the wrong location were excluded from the study. Fifteen mice fit this criteria.

Figure 6: Post injection imaging. Two weeks after injection the mice were imaged with 5x wide-field epifluorescence microscopy to confirm location and expression of injections. The vasculature is used as a guide for comparing the site of expression to the targeted site of injection given by the retinotopic map. Here, V1 is expressing GFP, shown in green, and the injected HVA is expressing tdTomato, shown in yellow. These locations correspond to the spots of fluorescence given by the map in Figure 2.

Histology

In preparation for histological analysis, mice were perfused with 10 mL 0.1 mM PBS followed by 40 ml of 4% paraformaldehyde (PFA) in PBS. The brain was removed and left to submerge in 4% PFA for 24 hours. Afterwards, 100 µm coronal slices were made using a Vibratome sectioning system (Leica). These slices were then mounted onto a glass slide with DAPI solution. DAPI labels cell nuclei, so it is brighter in places where cells are more dense. Since cortical layers vary by density, it is useful in distinguishing layer boundaries. Images of
tdTomato, GFP, and DAPI fluorescence at V1 and the HVAs were then taken with a 10x objective on a Zeiss LSM5 confocal microscope using Zen 2009 acquisition software (Figure 7). Three channels were used to capture the different fluorescent molecules. Both tdTomato and DAPI were excited by light at 561 nm, but tdTomato was seen through a long pass 580 nm cutoff filter and DAPI through a band pass 415 to 480 nm filter. GFP was excited by light at 488 nm and visualized through a band pass 495 to 555 nm filter. Exposure time, pinhole diameter, laser power, and gain were kept constant while taking images from each mouse for each experiment.

Figure 7: Images from confocal microscopy showing determination of lamina and labeling of cells. The eGFP in green on the left was used to identify the location of V1 in slice. Additionally, GFP labels layer V very strongly. Dapi staining also helps in finding layer boundaries by showing the outlines of layers II-III and IV. The tdTomato labels cells in V1 that are projecting to the injected HVA. Together these fluorescence channels help show how many cells in each layer are projecting to the HVA of interest.
Data Analysis and Statistics

Image analyses were performed using MATLAB (MathWorks), ImageJ (NIH), R (RStudio), and Excel (Microsoft). Confocal images were rotated in ImageJ so the pia was horizontal and then uploaded to MATLAB. Scripts specific to measuring number of labeled cells, average intensity of fluorescence, and total volume of expression were then designed for image analysis, and data from these scripts was then imported to Excel and RStudio for statistical breakdown and graphical representation.

Figure 8: MATLAB program used to count and localize cells. The counting function gives the tdTomato channel for each slice, and the movement through the slices is possible with the bars on the top and left. After clicking on all possible cells, the spots that are above a contrast threshold are recognized as neurons and represented as white dots in the mask output.
Retrogradely Labeled Cell Bodies

The layer specificity study required counting the number of labeled neurons in each layer of V1. A series of functions designed by Charlie Haas were then used to identify cells labeled with tdTomato in V1. Images were viewed in a graphical interface that allowed for moving between depths of the confocal z stack (Figure 8). In each image, labeled neurons were manually identified and if above a contrast threshold then they would be recognized as a cell by the program. After identifying all cells, a binary mask is created that shows neurons as white spots on a black background. Layer boundaries were then identified using DAPI stain, and in the mask these boundaries separated the white spots into the layer to which the cell belonged. This allowed for standardized identification and quantification of cells.

Extent of Local Expression

HVA injection sites were also analyzed for evenness of injection across cortical layers. Namely, the average intensity and volume of infection was compared between layers II-III and V and then related to the differences in V1 labeled cell counts between these two layers. Images of tdTomato fluorescence at the injected HVA were uploaded to MATLAB with the pia horizontal. A function was derived whereby the brightest coronal slice for each mouse would be identified and the 95th percentile of the pixel intensity values would be found. Twenty-five percent of this value would then be calculated and used as a lower threshold. All pixels above this threshold were considered infected. Coordinates corresponding to the layer boundaries of II-III and V were then found for each HVA and the amount of pixels above the threshold was calculated for each layer. The volume of infection was calculated by summing the pixels above the threshold in each slice and multiplying by a pixel-to-micron conversion factor to account for three dimensions. Average intensity was calculated by averaging the intensity of pixels above the threshold within
the layer coordinates. Ratios were calculated that divided each value in layer II-III by each value in layer V.

**Anterogradely Labeled Axons**

Analyses were also conducted to measure the relative intensity of axons projecting from V1 to the HVAs. Confocal images of the HVAs were cropped to include only the areas of fluorescent axonal arborizations. This cropping included both limiting x and y coordinates and selecting only ten images in each z stack that correspond to the inject site. The average pixel intensity for each HVA in each mouse was calculated for each channel, either green from GFP or red from tdTomato. Background fluorescence was then subtracted from these values by finding the average fluorescence in an area that was uninfected by virus. Afterwards, the red to green ratio was calculated for each HVA by dividing the average red fluorescence by the average green fluorescence. These values were compared across mice to look for similarities in HVAs that potentially belong to the same stream.

**Statistical Measures**

Each study required slightly different computations depending on the nature of the experiment. Multiple t tests were used when comparing the proportion of labeled neurons in each layer averaged across mice. These used the standard error to find if the difference in proportion in mice injected in different HVAs was greater than would be expected by chance. Similar t tests were planned for mice where axon projection intensities were assessed, but after processing only three were viable, with two injected in PM and one in LM. More mice injected in each HVA are necessary for a proper t test. Consequently, the base values were compared in each mouse in order to draw tentative conclusions.
When comparing the normalized counts, intensities, and volumes to each other in assessing evenness of HVA expression, regression was performed on three graphs with each plotting two of the measures. The R squared value was used as an assessor of the strength of the correlation, and the p value helped test for significance with lower than 0.05 being considered significant.

RESULTS

The overall goal of this experiment was to uncover anatomical features of the mouse primary visual cortex that could contribute to a stream organization. Laminar specificity of V1 is a key part of the anatomical organization of the primate visual cortex and there forms the initial segregation of visual information into dorsal and ventral streams (Shipp & Zeki, 1985; Shipp & Zeki, 1989). Finding evidence for laminar specificity in mice would provide additional anatomical basis for visual streams in this animal.

Additionally, we evaluated the multi target nature of projections from V1 to the HVAs. A V1 neuron synapsing onto multiple HVAs of the same stream could present a key anatomical feature for sharing information among the HVAs. This feature might hint at a fundamental aspect of sensory anatomical circuitry that helps constitute visual streams.

Anatomical Basis from Layer Specificity

Evaluation of Laminar Specificity: We set out to find if there are HVAs in the mouse that receive innervation from primarily one cortical layer of V1 in order to show a layer specificity that could account for streams. This was accomplished through a pairing of adeno associated virus (AAV) injections in the visual cortex of mice with the flex-tdtomato genotype. These viruses would label either retrogradely or anterogradely, depending on whether the virus binds to
kinesin, a motor protein that travels down the axon away from the nucleus, or dynein, a motor protein that goes up the axon towards the nucleus (Suomalainen et al., 1999). The virus AAV1.hSyn.eGFP was injected into V1, which caused anterograde labeling of V1 axons with eGFP, a green fluorescent protein. Afterwards, AAV1.hSyn.Cre was injected into one of three HVAs of interest: LM, AL, or PM. Four mice were injected in AL, five were injected in LM, and three were injected in PM The virus caused retrograde expression of the tdTomato gene built into
Figure 9: Distributions of V1 cells projecting to each HVA. (A) Raw layer counts for each individual mouse across the whole V1. Prefixes to the mouse names describe the HVA where Cre was injected. (B) Representative slices of V1 from each mouse. Red punctae are tdTomato-labeled cell bodies. Images are oriented with the pia on top. (C) Sum of raw layer counts added across mice injected in the same HVA. (D) Proportion of labeled V1 neurons in each layer averaged across mice injected in the same HVA. Used to control for the variation in labeling seen in B.

the genetic structure of this strain of mouse. As a result, V1 cell bodies projecting to the injected HVA were labeled with red fluorescence. This, combined with the DAPI stain, allowed for quantification of cells projecting to a particular HVA in each cortical layer (Figure 9A). Mice showed varying levels of expression and laminar distribution of labeled cells.

Mouse names indicate the strain of mouse and how projections from V1 to the HVAs were analyzed. Mice with GH or tdh designation were of the tdTomato strain while mice with CD or EB in their name were C57 wild type. Though the wild type mice were primarily used for analyzing the multi target nature of V1 pyramidal cells, they also had red-labeled cells in V1 that projected to a particular HVA. Consequently they were included for additional data regarding laminar specificity. These mice had flex-tdTomato virus and eGFP-expressing virus injected into V1. A retrograde traveling Cre was then injected into a particular HVA. The flex-tdTomato virus allowed for specific expression of tdTomato in V1 neurons that project to the injected HVA.

It is interesting that the tdTomato mice show greater background fluorescence. This may be because cells labeled in the wild type mice were inherently brighter, which required a lower exposure time and would then generate less background. Direct injection of flexed virus may insert more tdTomato genes into the V1 cells than are present in the tdTomato strains, which could lead to more transcription of the fluorescent protein and thus higher intensity. Additionally, the background fluorescence could be a real artifact reflecting the differences between the methods. Injecting into a flex-tdTomato mouse labels all neurons projecting to the HVA, which would label
more axons. These axons passing through V1 could create a small background fluorescence that is not seen in the wild type mice that only label V1 neurons projecting to the injected HVA.

Raw counts of neurons within each layer were attained by summing across mice injected in the same HVA (Figure 9C). In general, the most HVA-projecting cells were found in layer 2/3, whereas only a small minority resided in layer 4. For mice injected in AL there are 942 cells labeled in layer 2/3 while only 188, 490, and 316 cells labeled in layers 4, 5, and 6 respectively. Similarly, mice injected in PM show 815 cells labeled in layer 2/3 while only 69, 360, and 296 are in the other layers. Mice injected in LM show a slightly smaller difference between layer 2/3 and the deeper layers, with 867 cells labeled in layer 2/3 and 717 in layer V.

It should be noted that the cortical layers have different densities and sizes as well, with layer 4 the smallest and most dense while layer 2/3 is the largest and least compact. Despite these differences, labeled cells showed the same patterns when analyzing their density per unit volume as when simply looking at the raw counts. Consequently this density measure was disregarded when comparing injections in different HVAs.

Retrograde injections into an HVA cause a similar distribution of labeled cells, regardless of which HVA is injected. Generally the most cells are labeled in layer 2/3 while layer 4 contains the least labeled cells and layers 5 and 6 are intermediate. From the raw sums it might seem like LM receives a larger amount of inputs from layers V and VI. However, the differences in the efficacy of retrograde labeling across mice could skew results in favor of mice with above average expression.

To control for this, the proportion of cells in each layer out of the total amount of V1 labeled cells in each mouse was calculated. This gives a proportion for each layer in every mouse. These proportions were averaged across mice to give a normalized account of the labeled
cells in each layer for each injected HVA (Figure 9D). Normalized counts show similar trends as the raw counts, with the largest proportion of labeled cells being in layer 2/3 and the smallest in layer 4. For instance, for mice injected in AL, 49.7% of the labeled cells were in layer 2/3 while 9.8% were in layer 4, 25.4% were in layer 5, and 15.1% were in layer 6. Additionally, the proportion of cells in each layer is relatively similar across mice with different HVAs injected. For mice injected in LM layer 2/3 has on average 41.6% of the labeled cells while for mice injected in PM this layer 2/3 average is 56.4%. These values are well within the standard errors for the measures. Still, these standard errors are quite high, with a maximum of 16.7% for layer 2/3 of mice injected in PM. Variation in distributions of labeling among mice injected in the same HVA (Figure 9B) causes this large standard error, along with the small sample size. Our next goal was to determine whether there might be a systematic cause for this variation.

There are multiple potential experimental variables that might cause this variation, including injection damage, natural anatomical differences, or uneven diffusion and expression of virus. Slices in V1 with extensive damage were excluded, and the slices analyzed were relatively healthy, with well-labeled cell bodies (Figure 9B). Natural variation is possible but unlikely, given some of the extreme differences seen, such as the proportion of labeled cells in layer 2/3 of GH11 versus in layer 2/3 of CD3, which were both injected in PM. Consequently we investigated the evenness of virus expression as a possible source of individual distribution biases.

Analysis of HVA Infection for Layer Bias: One potential source of bias is in the distribution of expression through the layers of the higher visual areas. Injections were made at two depths, 250 µm and 500 µm, in an attempt to evenly express the virus through the HVA. Still, possible factors such as asymmetric diffusion or different neuronal expression capabilities
because of varying cell characteristics across layers might lead to uneven expression of Cre in the injected HVA. This uneven expression could potentially cause a bias in retrograde labeling of neurons in a layer of V1. Consequently, it was necessary to determine whether there are differences in expression across HVA layers related to the differences in cell labeling between V1 layers.

**Figure 10:** Analysis of Cre infection at the HVAs. (A) The brightest slice of the HVA from each mouse in the tdTomato (red) channel. Pia is oriented on top. (B) Graph of the log of the count ratio versus the log of the intensity ratio. The $R^2$ and p values are located in the top left. (C) The log of the count ratio graphed against the log of the volume ratio, with quantifiers again in the top left. (D) The log of the intensity ratio graphed against the log of the volume ratio, this time with quantifications in the top right.

Cre injection sites were located and each slice encompassing the site was imaged (Figure 10A, representative slices). Initial inspection shows generally even expression across layers, with
maybe slightly stronger expression in layer 2/3: generally the intensity is consistent but fading in the deeper layers and at the edges. Also, while the same volume of virus was injected into each mouse, the HVAs have different widths of expression of tdtomato. This may be a result of differing injection efficiencies or varying diffusion patterns.

This analysis was only possible in flex-tdtomato mice. The HVA cells in wild-type mice do not contain the tdtomato gene to be activated for expression, so there is no fluorophore to mark Cre expression in cells residing in the HVA. Consequently, the injection sites for the wild-type mice could not be analyzed for the spread of Cre infection and expression.

There are several possible methods for using the expression in the HVA for assessing the probability of V1 retrograde infection. Intensity level should be proportional to the level of protein expressed and the amount of virus entering cells at that area. Consequently finding average pixel intensity of the injection site can be one useful method. However, the amount of fluorescence in the HVA could be unrelated to how many V1 cells are retrogradely labeled. Additionally, axons traveling through the tissue would also contain fluorescence. To account for this, volume of injection in the injected HVA could be a more accurate measure of the probability of V1 cells being retrogradely labeled.

Consequently, both volume and intensity measurements were calculated for layers 2/3 and 5 of the HVAs. Average intensity was calculated by taking the mean intensity of all pixels above a certain value determined to be the minimum intensity for infection (see Methods). Similarly, total volume could be calculated by summing the volume of all pixels above this threshold. These calculations gave measures for the expression of virus at the HVA injection sites.
A correlation between the laminar measures of expression in the HVA and the V1 cell counts might explain the observed variability in the proportion of cells in each V1 layer that project to a particular HVA. To normalize our expression values we divided the measure in layer 2/3 to that in layer 5. This allowed us to compare measurements for expression across layers. The normalized expression values are graphed against a normalized count ratio in order to see if unevenness in expression could lead to a labeling bias.

The normalized count and intensity values show no significant correlation (Figure 10B). Though there seems to be a general positive trend, the R squared is only 0.07, which does not indicate a strong relationship. The two mice with the highest values for both ratios (count and intensity) were injected in area PM, with one at (6.8, 1.0) and the other with (5.2, 0.98), suggesting there is not a strong relationship between the two variables. Analyzing intensity gives no evidence that uneven expression leads to biased cell counts.

However, the range of values of normalized intensities is very small. The highest ratio is 1.0 and the lowest is at 0.79, though this is an outlier and the next highest is 0.90. This might indicate that there is not a significant difference in the expression of the virus through the tissue, and that the virus is effectively even by measure of the average intensity.

The normalized volumes were also used as a measure of evenness of expression. These values were graphed against the normalized counts, which again showed no relationship and an R squared of -0.22 (Figure 10C). This relationship is stronger than that between intensity and count, but it is in the opposite direction. Although volume could potentially be a better indicator of virus expression, the intensity of the threshold did not necessarily correspond to the minimum amount of virus necessary to cause retrograde labeling.
Consequently, the volume of the fluorescence might be more a measure of the concentration of the virus after it was injected into the brain, with a small volume meaning a greater concentration of virus and more effective retrograde labeling. This might not be reflected in the intensity if the tdTomato reaches a peak expression that is limited by the availability of the tdTomato gene, so any additional Cre will cause no intensity increase. Still, the range between the volume ratios is from 0.77 to 1.6, which is small compared to the range between the normalized counts. As a result the very weak relationship observed can be coincidental.

The normalized ratios were then compared to each other to test their validity as measures of expression. They also show no correlation (Figure 10D), which may indicate that one or both of them are not measuring expression of virus. If both of them were accomplishing this then there would be a strong correlation since they should both change with correlation. Either they are negatively correlated, whereby diffusion of the virus and a larger volume might cause a lower density of cells expressing and therefore lower intensity, or they are positively correlated, so that a high volume indicates a large amount of the virus introduced to the HVA and therefore higher expression rates and intensity. Though there does seem to be a downward trend with an $R^2$ of -0.07, overall the lack of a relationship shows that one or both of the measures are not a good indicator of expression strength. Alternatively, the lack of variability across layers might be insufficient to show a trend for or against biases as a result of injection distribution.

Overall, the absence of a strong relationship between the normalized count and both the normalized intensity and volume supports that there was little bias from unevenness of expression. It is also doubtful that the injections are uneven enough to be significant given the small range across the volume and intensity ratios. Analyzing HVA expression validates the null results of the layer specificity experiment, thus supporting the finding that HVAs have the same
proportions of cells from each layer projecting to them. This lack of layer specificity fails to offer support for an anatomical basis for streams in mice.

**Anatomical Basis from Multi Target Projections**

*Assessing the Prevalence of Multi Target Projections:* In order to investigate other aspects of anatomy that might contribute to stream organization, we assessed the degree to which individual neurons in V1 project to multiple HVAs. A V1 neuron projecting to multiple HVAs would present a feature for sharing information across HVAs, which would support a stream organization among these areas.

Investigation of these multi target projections was accomplished through dual injections of fluorophore-expressing viruses into wild-type mice (Figure 11A). An injection was made into V1 consisting of a combination of eGFP virus and flex-tdtomato virus. The eGFP virus would cause anterograde expression of green fluorescence in V1 cells. This fluorescence would ideally label all V1 neurons and consequently enable visualization of the baseline rate of V1 projections to each HVA. The second virus, flex-tdtomato, inserts the tdTomato gene into the V1 neurons it infects. This gene is silent unless there is co-expression of Cre in the cell, which will activate the gene so it can be transcribed. To enable this expression, Cre-expressing virus was injected into an HVA of interest, either LM, AL, or PM. This virus causes retrograde expression of Cre, which then causes transcription of red fluorescence in the V1 neurons infected with flex-tdtomato. Consequently, V1 axons projecting to a particular HVA were labeled in red, while all V1 axons were labeled in green (Figure 11B).

Since only the cells that project to the targeted HVA will express tdTomato, any red fluorescence in an uninjected HVA would be a result of neurons with multiple target projections. Moreover, if multiple target projections are identified, the patterns of shared targets could tell us
about the potential organization of these projections into streams. For instance, two dorsal stream areas (such as AL and PM) would be more likely to have shared projections than a dorsal and ventral stream area (such as LM and AL). A stream organization would be supported by HVAs in the same purported stream as the injected area having higher red fluorescence than the other HVAs. For instance, if AL was injected with Cre, then AL and PM would be expected to have higher red fluorescence values than LM.

**Figure 11:** Red and green fluorescence intensity values. (A) Diagram of the experimental design. Retrograde cre-expressing virus injected into one higher visual area of a wild-type
mouse. A mixture of anterograde tdTomato and eGFP virus injected into V1. (B) Representative slices of the three main HVAs of interest from a single mouse. Red channel on the left and green channel on the right. (C) Average intensity values for both channels in the injected higher visual area versus the non injected visual area. (D) Red to green ratios for each HVA for each mouse. Colored abbreviations underneath indicate the HVA that was injected with retrograde cre virus.

First, though, we analyzed the data as a whole to look for evidence of any multi target projections from V1 to the HVAs. The raw red and green fluorescence values were averaged across injected and non-injected HVAs after subtracting the baseline (Figure 11C). Red fluorescence was compared to assess multi target projections, while the green fluorescence was used as a positive control. The average red fluorescence values for injected and non-injected HVAs are 916 ± 96.9 and 924 ± 86.3, respectively. The average green intensities are 1751 ± 344.3 and 1328 ± 219.6 for the injected and non-injected, and these close values support the validity of the experiment. The similarity of the red intensities suggests a multi target projection pattern where there are a significant number of V1 neurons that project to multiple HVAs.

We were surprised by the similarity of red fluorescence intensity across injected and uninjected HVAs. These results support the abundance of multi target projections more strongly than anticipated. However, the baseline projection density from V1 to each target is not the same. Consequently it is not fair to make a direct comparison across these areas. Instead, we devised a way to quantify the fraction of projections to an area that is accounted for by shared projections using the green fluorescence to normalize the red. The red to green average intensity ratio gives a measure of the proportion of all projections in the analyzed HVA that are from cells also projecting to the HVA injected with Cre.

These red:green ratios were calculated for each HVA in each mouse (Figure 11D). For each mouse we would expect to see the highest red:green ratio in the injected HVA since it should have all projections from V1 labeled with both red and green, while the other HVAs
would have all projections from V1 labeled with green and only a fraction with red. We found that there were many mice where the injected HVA did not in fact have the highest red:green ratio. For instance, CD2 was injected in LM yet showed a 0.50 red:green in LM and a 0.62 red:green in PM. Factors related to the quality and nature of the injections and infection of the cells were then investigated for possible explanations before a final analysis of the data.

*Baseline Organization of Projections to the HVAs:* The raw green fluorescence offers a good control for showing the connectivity from V1 to the HVAs and ensuring it is in line with previous research. Namely, previous investigations of the density of connections from V1 to the HVAs revealed that the strongest inputs were to LM, followed by AL and PM. Deviation from these ordinal relationships might suggest either damage from the injection pipet or problems with virus expression.

To assess this connectivity, we normalized the average green fluorescence in each mouse by dividing by the average intensity in LM, since this HVA should have the most projections from V1 and therefore the highest green intensity. These normalized values were compared to the connectivity levels found in past work (Figure 12A). Based off of these previous studies, an expected green distribution would show LM having the highest average intensity, with AL having an intensity 67.5 % of LM’s and PM with an intensity 55 % of LM’s. The data shows three of the mice having this expected V1 connectivity, while the other three show unexpected
Figure 12: Exclusion of mice showing abnormal connectivity. (A) Comparisons of normalized green intensity distributions. Each mouse is shown in a different color with past work from Burkhalter in dark green. Any mice not conforming to the Burkhalter plot were excluded. (B) Comparison of colocalization in mice with the unexpected green distribution (left) and the expected green distribution (right). The inset shows a representative sample of cells from each
mouse, with the cells on the left having a lower rate of colocalization than the cells on the right. (C) Comparisons of the red:green ratios in each mouse with the expected green distribution. HVAs are marked by color and purported streams are marked by shape.

intensity distributions. For instance, the average green intensity in CD2 in AL was 134.7 % of that in LM, which would suggest that AL has a stronger connectivity with V1 than LM.

One possible reason for the unexpected projection densities to the HVAs might be related to infection of V1 cells. In the experimental design, all V1 cells are labeled with green while V1 cells projecting to the injected HVA are labeled with red. Therefore, all cells labeled with red should also be labeled with green. Absence of this colocalization might indicate that the viruses interfere with each others’ expression, which could create problems in interpreting the red:green fluorescence intensities in the HVAs for understanding the relative density of multi target projections.

Slices were examined for colocalization of tdTomato and GFP in V1. Instances of non-colocalization were more apparent in mice that displayed unexpected distribution of green intensities in the HVAs (Figure 12B). In CD2 there are multiple instances where there is a red-labeled cell that is not also labeled with green. These instances also occur in CD3, a mouse showing expected green intensity distribution, but it is much more common to see red-labeled cells that are also labeled with green, as seen in the inset.

Guided by the measures of connectivity via the normalized green values, we excluded mice showing unexpected green distributions. With the remaining mice we compared the red:green values across HVAs (Figure 12C). HVAs were also grouped into streams as purported by previous work, with AL and PM in the dorsal stream and LM in the ventral stream. The HVAs in the stream injected showed higher red:green values than the HVAs in the other stream. Although the injected HVA did not always have the highest ratio, HVAs in the injected stream as
defined by Wang et al., 2012 had a higher red:green ratio than HVAs in the other visual stream. For instance, in CD6, which was injected into PM, PM and AL have red:green of 0.96 and 1.06, respectively, and LM has a red:green of only 0.86. This might suggest some stream-specific pattern of multi target projections.

Overall, the data suggests that multi target projections from V1 to the HVAs are a prominent anatomical feature of the mouse visual cortex. Many of the mice do not display the distribution of green fluorescence that would be expected from the known connectivity between V1 and the HVAs. These unexpected distributions might be from a lack of virus colocalization in mouse V1 cells. Still, when analyzing the mice with an expected green distribution we find that HVAs in the injected stream have a slightly higher red:green than the other HVAs, which offers some support for stream-specific multi target projections.

**DISCUSSION**

The goal of this study is to understand how the anatomy of the mouse visual cortex might contribute to a stream organization similar to that seen in primates. This will help us understand visual processing as a whole and will help provide a context for future experiments in mice. Structure is closely related to function, and better understanding the anatomy of the mouse visual cortex might help reveal mechanisms involved with visual processing in context. Past work has found elements of visual streams in the connectivity (Wang et al., 2012) and development (Smith IT, Townsend, Huh, Zhu, & Smith SL, 2017) of the mouse visual cortex. Here, we used fluorescent labeling via viral injections to investigate anatomical features of projections from V1 to the HVAs that might contribute to a stream organization. We looked at two aspects
specifically: laminar specificity of V1 cells projecting to the HVAs and the extent and structure of neurons in V1 synapsing onto multiple HVAs.

These experimental questions were informed by previous studies in primates showing laminar specificity in projections to different streams between the layers of the primary visual cortex (Shipp & Zeki, 1985; Shipp & Zeki, 1989). A stream organization in the mouse visual cortex would be most strongly supported by finding both that HVAs receive projections from primarily one layer of V1 and that individual V1 neurons synapse onto multiple HVAs all within the same purported stream. However, a positive finding in either question would show some degree of stream organization, and a lack of anatomical support for visual streams might indicate an important evolutionary development in the history of the primate cortex that can help in our understanding of how visual processing influences behavior.

*Labeling of Primary Visual Cortex Cells*

Labeled V1 neurons projecting to an HVA showed patterns that would be expected from past research. For instance, layer 4 consistently showed the lowest amount of labeled cell bodies, which is consistent with past research finding that most thalamic inputs to V1 synapse onto layer 4 (Sun, Tan, Mensh, & Ji, 2016). If the primary purpose of layer 4 is to receive information and distribute it throughout V1, then it would follow that it does not have a lot of projections to the HVAs. Additionally, although V1 layers were found to differ slightly in response properties (Niell & Stryker, 2008), it was found that these layers do not differ according to the specific spatiotemporal properties inherent to the HVAs (Glickfeld et al., 2013). This lack of functional specialization generally supports the absence of laminar specificity separating the streams of the primate visual cortex.
Despite this support of the results, there were some errors in carrying out the procedure that may have affected the outcome of the experiment. The viral injections necessary for the experiment investigating laminar specificity required a great deal of care and precision. Consequently, there were many mice with unusable data as a result of primarily either damage to the visual cortex or a lack of virus expression. Injections certainly improved over the time course of the study, but some mice still have sparse labeling or uneven distribution of labeled cells that is inconsistent with the overall results and is most likely too extreme to be due to natural variation between mice. These contributed to the large error bars seen in the data. Damage to the brain from the injections is one possible explanation. However, care was taken during the durotomy and crafting of injection pipet to minimize the death of cells at the site of entry. Additionally, the unevenness of labeled cells is seen in mice where there is no visible damage, and there are normal distributions in mice with significant cell death.

In addition to damage from the injection, the concentration and distribution of virus in the HVAs might have caused uneven expression in the area and layer bias in the overall cell labeling. Previous work leading up to these experiments involved considerable adjustment of optimal concentration of virus for V1 labeling, so it is not likely that the concentration negatively affected the diffusion and expression of virus. Additionally, injections were made at 250 and 500 micron depths in order to prevent uneven expression. Most encouragingly, there was no association between intensity or volume of the HVA injection site and the cells labeled in V1. Still, it is unclear how efficiently these measures quantify expression.

*Issues Regarding Quantification of Expression*

Two measures were used for characterizing expression through the layers of the HVAs: average intensity and volume of infection. We assumed that average intensity would be related to
the amount of protein transcribed, with a higher intensity showing greater fluorescence. A larger amount of protein was interpreted to mean that there was more Cre in the cell, which would therefore give the Cre a higher probability of retrogradely infecting any V1 cells projecting to the injected HVA. Consequently, a higher intensity in a layer of a HVA might cause a labeling bias in V1 if cells in V1 project primarily to a corresponding layer of their target HVAs.

There are some potential issues with this model. First, intensity of tdTomato fluorescence in the HVA might not relate to the probability of virus being taken up retrogradely by V1 cells. Using the intensity as a measure is subject to variables unrelated to how the virus enters V1 cells. For instance, axons traveling down the HVA back towards V1 would cause a higher intensity in the lower layers that they pass through, but this does not mean that the virus would be more effective in these layers. Finding the volume of the HVA injection site was also used to supplement some of these issues, but these tests yielded similar results.

Tropisms of V1 cells in different layers could also affect the retrograde infection abilities of the cells, thus biasing the results. For instance, GFP shows high intensity in layer 5 cells of V1, which have cell bodies that are noticeably larger than those in the other layers. Perhaps these cells have a faster metabolic rate because of their large size, which might require faster, more abundant resources for transcription that would lead to more fluorescent protein and a brighter intensity. Alternatively, the large cell bodies might indicate that all parts of the cell are larger, so their axons and synaptic boutons have more surface area and a higher probability of infection than smaller cells. Tdtomato expression does not show a higher intensity in layer 5, but probability of V1 infection could still be affected by greater surface area. Additionally, since infection of virus depends partly on extracellular receptors (Thoulouze et al., 1998), differing
amounts or types of receptors across cortical layers could cause a bias in the probability of
infection.

Despite the findings of no relationship between V1 counts and the measures of
probability of infection, it should be noted that there may be effects specific to each HVA, and
since here all of the HVAs were analyzed as a whole then these might have been missed. Using
more mice might make an HVA-specific analysis possible. Additionally, injection of dextrans
into primarily one layer of the cortex could serve as a more direct method of elucidating how an
uneven injection would affect the probability that V1 cells in each layer are labeled.

**Axon Fluorescence in the Higher Visual Areas**

Examining multi target projections in the mouse visual cortex showed that many neurons
in V1 project to multiple higher visual areas. There was significant red fluorescence in HVAs
that were not injected with Cre. Additionally, the green fluorescence values in the HVAs proved
to be an efficient method of assessing the injections for quality and accuracy of expression. Last
it was found that HVAs in the same purported stream as the injected HVA show higher red:green
than the other HVAs, which might support a slight stream-specific multi target projection
pattern.

Still, various complications arose when quantifying the amount of projections to the
HVAs using fluorescence from injected virus. Many glia were labeled in some of the injections,
which interfered with quantification of fluorescence. This was relatively easy to control for by
excluding slices with glia labeling or when necessary cropping sections with glia. Additionally,
there were a lot of mice that did not show adequate expression for visualization of axonal
projections. There were also HVAs in usable mice that did not show green fluorescence, even
though theoretically all HVAs should be showing some level of GFP fluorescence dependent on
the amount of projections they receive from V1. Damage to certain HVAs also prevented some quantification. Areas with these problems were generally excluded from the study and the six mice used all had healthy, visible areas that were capable of comparison.

Use of fluorescence of V1 projections might also not be the most optimal way to assess the connectivity of V1 neurons with targets in multiple HVAs. Fluorescence intensity may be due to the inherent properties of cells from different layers, which might not necessarily be related to strength or quantity of connections. Additionally, fluorescence does not indicate the strength of the synapses between V1 cells and the HVAs. This question is more physiological and outside of the scope of this anatomical study, but it comes up when interpreting the data.

One major issue was the disagreement of connectivity implied from green fluorescence with past literature. The proportion of green fluorescence in each HVA should be generally the same in each mouse because GFP labels all V1 cells, which will have similar connection patterns overall across mice. Also, this connectivity should be concordant with previous work showing that V1 projects most to LM, with AL and PM following and the rest of the HVAs receiving the smallest amount of projections. This pattern was not observed in half of the mice analyzed, which questions the validity of the green fluorescence for quantifying projections from V1 for these mice.

We were unsure the cause for these surprising patterns of fluorescence intensity. One possible explanation is that the expression of one virus interferes with the expression of the other, which could cause the lack of colocalization observed in V1. This might cause a decrease in green fluorescence in cells that are projecting to the injected HVA, or it might cause the inhibition of red fluorescence in HVAs, such as LM, that have strong projections from V1. The directionality of the bias depends on which virus outcompetes the other. If red is dominant to
green, then we would expect the fluorescence of green in the injected HVA to be lower than expected. This is seen in some mice, such as CD2, but it is not seen in all. For instance, CD1 was injected in AL yet also had the highest green fluorescence in AL. Consequently, it could be the case that it is common for green to outcompete the red. From our data it is only possible to tell places where red prevented expression of green, since all V1 cells should be labeled with green.

The possibility still remains that the stochastic manner of infection caused some cells to be labeled with only one virus while others are labeled with both. This could be tested by performing injections of anterograde eGFP virus into V1 and comparing the distributions of green fluorescence in these mice to those in mice with dual injections. Finding distributions closer to those in Wang et al., 2012 in this experiment would suggest that the viruses interfering with each other to prevent colocalization could be a source of some of these unexpected observations. If it happens that the viruses do not interfere but simply do not colocalize because of probabilistic chance, then the concentrations of the viruses injected should be adjusted to increase the chances of colocalization.

**Interpretations and Future Directions**

The final results offer promising yet extremely tentative evidence for an anatomical basis for streams in the mouse visual cortex. This basis is not observed in laminar specificity as the three HVAs LM, AL, and PM seem to receive projections from generally the same proportion of cells from each layer. This presents an interesting evolutionary development between the common ancestor of the mouse and primate and the eventual formation of our visual cortex. Future work could attempt to understand how the layer specificity of the primate visual system contributes to visual processing behaviorally in a way that is not present in that of the mouse. Additionally, work in the future can look at cell-type specificity of neurons in V1, as layer 5 V1
neurons of slightly different genotypes have been shown to have different projection patterns (Kim et al., 2015). This kind of organization might have been a prelude to the laminar specificity observed in the primate cortex.

An interesting result from the laminar specificity study is that an abundance of neurons in layer 2/3 project to the HVAs, while very few neurons in layer 4 show the same projection pattern. Layer 4 receives input from the thalamus, so it most likely projects primarily to other layers of V1 to distribute information throughout this structure. The abundance of labeled neurons in layer 2/3 is potentially interesting, as cells in this layer have been shown to be capable of high plasticity (Benoit, Ayoub, & Rakic, 2015). Additionally, this layer shows low orientation tuning width (Nie\ll & Stryker, 2008). Consequently, HVAs might receive a proportionally large amount of information from this layer in order to supplement their response characteristics relating to motion, especially when learning shapes of new objects. Future studies could investigate if the distribution of proportions for V1 cells projecting to an HVA in each layer changes if the mouse is taught to respond to a stimulus that has response characteristics of the HVA in question.

The strongest anatomical support for streams is from data examining V1 neurons with projection targets in multiple HVAs. Of the three mice with normal patterns of green fluorescence, the HVAs in the injected stream had a higher red:green ratio than those in the non-injected stream. Although there are only three mice and the differences are subtle, it provides evidence for an anatomical bias that might show the beginnings of stream organization. This anatomy could provide a mechanism for sharing information among the HVAs, and future studies can look for similar networks of structures in primates. In mice also, future work can be
focused on assessing the response properties of neurons that project primarily to one purported visual stream.

The results from this study might be challenging to reconcile with previous work. It was shown that terminal boutons of V1 neurons share the same response properties as the HVA in which they synapse (Glickfeld et al., 2013). If the response properties are the same throughout the neuron, then this may pose a problem for V1 cells that project to multiple HVAs. This would especially be the case for neurons projecting to AL and PM, since PM responds to slow-moving stimuli while AL’s response properties correspond to fast-moving stimuli. It could be that neurons that project to multiple HVAs have intermediate response properties. This could be accomplished through a similar experiment using a calcium indicator such as flex-GCAMP instead of flex-tdtomato to measure the functional properties of neurons projecting to multiple HVAs. It could be expected that these neurons encode receptive fields with intermediate response properties.

Another alternative might be that multi-target cells participate in antagonistic inhibitory circuits. This mechanism might be used to isolate cells projecting only to one HVA and have response properties matched to that area. Extreme stimuli, such as very slow or very fast, might thus recruit strong inhibition, thereby sharpening the receptive fields and making the stimulus more salient. This might be accomplished by a class of V1 interneuron activated by neurons that project to a single HVA and have more extreme response properties. A system such as this would allow for a sharing of information across HVAs while activation is still weighted for HVAs with response characteristics matching a given stimulus. A physiological experiment finding an IPSP in response to an extreme stimulus in a V1 cell body with intermediate response properties is one way future studies might investigate this hypothesis.
Still, it would be useful to take a step back and consider the framework of visual streams in a general evolutionary context. Visual streams are partially a construct for helping us understand parallel processing in the higher visual system. Even in the primate there is some debate as to whether these streams constitute two completely independent parallel processing pathways (Schenk & McIntosh, 2010), partly because of the high level of interconnectivity between areas in different streams. Consequently it might not be useful to broadly apply the stream model to all animals with visual cortices slightly resembling that of the primate.

Additionally, though most of the literature assumes the seemingly parallel organizations of the mouse and primate cortex are homologous (Homman-Ludiye & Bourne, 2014), oftentimes citing Wang et al. (2012) for evidence of streams in the mouse cortex, it is possible that the structures are analogous and developed from different environmental pressures yet appear similar. This might cause differences in fundamental features that would be important to elucidate before drawing parallels. There is evidence that the regions are homologous, though, with some of the strongest being that mole rats, who are effectively blind and live in the dark, have defined V1 and V2 areas (Matsunaga, Nambu, Iriki, & Okanoya, 2011). This shows a conservation of features of the visual system seen in the primate in a rodent species that would not have had environmental pressures to develop them, though it is possible they could have been developed in previous rodent species. However, the vestigial nature of these structures in mole rats cautions against the use of other species for studying the processes through which humans use vision. Just because an anatomical structure is present does not mean it contributes significantly to perception or behavior.

Regardless, there are still many complex anatomical features of the mouse visual cortex that can be investigated in order to help us understand fundamental features regarding the
nuances of visual processing. Among these features are multi target projections, which have a lot of potential for organizing information from V1 into separate streams. Future studies should look at the response properties of neurons projecting to multiple streams to observe if they encode similar intermediate properties or maybe even physical stimuli that we have not characterized. Eventually, future studies should also examine the extent of multi target projections in the primate visual cortex to see if they also assist in sharing information among HVAs of the same stream in this organism or if it is a feature specific to rodents. These studies could reveal basic features of visual processing and show how anatomical specializations in our cortex allow for a distinctly human visual experience.
References


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