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Developing a System to study Multimodal Association of Stimuli in Mice

MSc Thesis

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Ανάπτυξη Συστήματος για τη Μελέτη Πολυτροπικής Συσχέτισης Ερεθισμάτων στο ζωικό μοντέλο των Μυών

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1. Abstract

Associating various sensory information to visual objects is a fundamental cognitive process that enhances the ability to recognize objects and improves memory performance. Mice are capable of invariant object recognition in their natural environment, however the associated changes of object manifolds along the cortex remain widely unknown. We suggest a new approach by establishing multimodal association of stimuli to determine if there is an overlap between neuronal populations who respond to different properties of the same object. Specifically, the question we are trying to address is whether the manifold of an object is formed in V1 area, while the mouse is exposed to an associated odor, giving us a chance to better identify which subgroup of neurons are actually responsible for object recognition. Using home cage training, we examined the ability of two different strains of mice (C57BL/6J & Thy1-GCaMP6) to associate a visual object with an odor. We found differences between strains during the behavioral training while proposing three different experimental paradigms (Go/NoGo, 2AFC, M2S) in order to conclude which is the most suitable. Furthermore, we finely tuned several different parameters that play a significant role in automated behavioral training. Overall, it was challenging to establish such an association while ensuring that the animals consider both stimuli to make decisions.

Keywords: multimodal; primary visual cortex; object recognition; mental imagery

1.1 Περίληψη

Η συσχέτιση διάφορων αισθητηριακών πληροφοριών με οπτικά αντικείμενα αποτελεί θεμελιώδη γνωστική διαδικασία που βελτιώνει την ικανότητα αναγνώρισης αντικειμένων και ενισχύει την απόδοση της μνήμης. Οι μύες έχουν την ικανότητα να αναγνωρίζουν αντικείμενα στο φυσικό τους περιβάλλον, ωστόσο οι συναφείς αλλαγές στις επιφάνειες αντικειμένου στον κατασκευασμένο 3D χώρο (object manifolds) κατά μήκους του φλοιού παραμένουν ευρέως άγνωστες. Προτείνουμε μια νέα προσέγγιση, εγκαθιδρύοντας πολυτροπική συσχέτιση ερεθισμάτων, με στόχο να καθορίσουμε αν υπάρχει ανατροφοδότηση μεταξύ νευρωνικών πληθυσμών που αντιδρούν σε διαφορετικές ιδιότητες του ίδιου αντικειμένου. Συγκεκριμένα, η ερώτηση που προσπαθούμε να απαντήσουμε είναι εάν η επιφάνεια ενός αντικειμένου στον 3D χώρο (object manifold) διαμορφώνεται στην περιοχή V1, όταν ο μυς εκτίθεται στην συσχετιζόμενη με το εκάστοτε αντικείμενο μυρωδιά, έχοντας την ευκαιρία να εντοπίσουμε καλύτερα ποια υποομάδα νευρώνων είναι πραγματικά υπεύθυνη για την αναγνώριση του αντικειμένου. Εξετάσαμε την ικανότητα δύο διαφορετικών στελεχών μυών (C57BL/6J & Thy1-GCaMP6) να συσχετίζουν ένα οπτικό αντικείμενο με μια μυρωδιά. Βρήκαμε διαφορές μεταξύ των στελεχών κατά τη διάρκεια της συμπεριφορικής εκπαίδευσης σε τρεις διαφορετικές πειραματικές διατάξεις (Go/NoGo, 2AFC, M2S) με σκοπό να συμπεράνουμε ποια από αυτές είναι πιο κατάλληλη και αποτελεσματική. Επίσης, βρήκαμε τις κατάλληλες ρυθμίσεις διάφορων παραμέτρων που απαιτούνται στην αυτοματοποιημένη συμπεριφορική εκπαίδευση μυών. Τελικά, η καθιέρωση μιας τέτοιας σύνδεσης, εξασφαλίζοντας παράλληλα ότι τα ζώα λαμβάνουν υπόψη και τα δύο ερεθίσματα για τη λήψη αποφάσεων αποδείχθηκε πιο δύσκολο και χρονοβόρο από ό,τι περιμέναμε.

Λέξεις – κλειδιά: πολυτροπικός, πρωτογενής οπτικός φλοιός, αναγνώριση αντικειμένου

1.2 Acknowledgement

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The completion of this study could not have been possible without my parents, who supported me financially throughout my university studies and my friends, who supported me emotionally throughout this roller-coaster ride.

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2. Introduction

Research in neuroscience increasingly relies on the mouse animal model due to its genetic tractability, brain atlases and numerous methods available to perform large scale recordings. Mice are an ideal species for studying perception, as they quickly learn to perform tasks based on a plethora of stimuli, including but not limited to olfaction and vision.

It is well established that many behaviors of mice, including learning and memory, navigation, etc., are closely associated with the sense of olfaction, thus it is considered their predominant sensory modality with which they process information about their environment. Contrary to past preconceptions, mice make major use of vision as well. Their visual cortex consists of at least 10 retinotopic areas. Most importantly, the division of labor across these areas and other general principles of visual function are likely to be conserved across species (Wang et al., 2011).

The visual cortex of the mouse has a lot in common with other species, as it bears the typical 6-layered structure, retinotopic organization and a plethora of excitatory and inhibitory neuronal subtypes. However, mice do not have a fovea, a highly specialized area that corresponds to 1% of the retina in primates and is responsible for high contrast and acuity (Huberman & Niell, 2011).

2.1 Cross-modal Integration & Mental Imagery

Real objects can be identified using different sensory systems. Thus, associating multiple sensory information with visual objects is an important brain process that can facilitate object recognition and memory performance. This cross-modal integration is constructed in a way that items can be represented both as a whole and as a subset of cross-modal features. In addition, the learning processes should account for plasticity – stability trade-offs, by shaping relevant new cross-modal associations, while actively ignoring irrelevant information and maintaining prior memories. Consequently, the development of cross-modal memories becomes a dynamic procedure that depends on experience across long time periods (Li et al., 2019). Nonetheless, neural mechanisms that connect sensory features during learning are largely unknown.

Mental imagery is a term used to refer to representations and the accompanying experience of sensory information without a direct external stimulus. They are recalled from memory and are not necessarily voluntary; associations also can trigger mental images. Brain imaging studies in humans have showed that neural representations of mental and perceptual images resemble one another as early as the primary visual cortex (V1) (Naselaris et al., 2015). Evidence suggests that mental imagery shares processing mechanisms with like-modality perception. In other words, mental imagery could function much like afferent sensory perception (Pearson et al., 2015).

Taking this information into account, the following question comes quickly to mind: will a mental image of an object be formed as early as V1, if the subject is exposed to associated sensory information from another modality? There are no studies examining mental imagery in mice to our knowledge. However, we propose the mouse animal model to be a good system to study mental imagery, as it is possible to train them relatively quickly to perform well in multisensory tasks.

2.2 Primary Visual Cortex & Mental Imagery

Historically, the study of mental imagery faced challenges due to practical and theoretical issues. Methodological limitations arose from the fact that mental imagery is a private experience, making it difficult to conduct certain mechanistic investigations. Additionally, during the latter half of the 20th century, behaviorism became dominant in psychology, discouraging the examination of internal mental processes. The combination of methodological constraints and the influence of behaviorism is primarily responsible for the limited research on mental imagery compared to other related subjects like visual attention and visual working memory (Pearson, 2014).

The primary visual cortex, known as area V1, is distinctive both in terms of its location in the brain and the visual information it encodes during visual perception. It holds a special anatomical position, as it serves as the gateway for retinal information into the cortex. It receives more direct connections from the lateral geniculate nucleus than any other part of the visual cortex. Nonetheless, during mental imagery, its proximity to the retina does not confer any special place. The exact source of mental imagery remains unknown, but it is likely that structures involved in memory encoding in the medial temporal lobe (MTL) and executive functions in the prefrontal cortex play a crucial role. Additionally, area V1 is unique in its representation of basic visual features. Models of visual perception that focus on the flow of information treat these low-level features as the fundamental building blocks of object representation. On the other hand, feedback models view them not as the foundation for constructing object representations but as a tool for verifying predictions about the objects in the immediate environment (Rao & Ballard, 1999).

The significance of V1 during mental imagery is probably linked to its topographical organization, which allows it to make explicit and accessible higher detail about objects, such as certain geometric properties that may only be implicit in long-term memory representations. In simpler terms, the role of V1 in mental imagery may be determined by the kind of inferences it enables us to draw from a mental image. For example, if we want to determine whether a dog has a pointed or floppy tail, we may need to incorporate a V1-like representation as part of our mental image. But if we simply want to assess whether a horse is larger than a cat, we may not require the involvement of area V1, while representations in various visual and parietal areas with topographical mappings might be sufficient. This concept aligns with findings suggesting that the extent of V1 activation during mental imagery depends on the specific task at hand (*The Case for Mental Imagery - Stephen M. Kosslyn, William L. Thompson, Giorgio Ganis*).

2.3 Multisensory Integration & Attention

Multisensory integration involves the coordination of information from different sensory modalities (vision, audition, olfaction, touch), affecting how these inputs combine to create a unified perceptual experience. Groundbreaking research investigating the response patterns of single neurons in anesthetized animals identified several key stimulus-driven factors, most notably the temporal and spatial concordance of cross-sensory inputs, as major determinants for multisensory integration (Meredith, 2002).

Attention encompasses mechanisms that determine the selection of specific sensory inputs, objects, thoughts, or actions from a range of possible stimuli. This selection can be based on an

individual's goals (top-down) or by a salient stimuli (bottom-up). Interestingly, temporally and spatially aligned sensory inputs from different modalities are more likely to attract attention, suggesting that multisensory stimuli are more captivating. Studies have shown that multisensory processes can capture attention in a bottom-up manner, indicating that multisensory integration operates automatically and before attentional selection processes (Van der Burg et al., 2008). However, in different situations, top-down directed attention can impact multisensory integration processes for specific combinations of stimuli within the environment (Van Ee et al., 2009).

A well-received review paper proposes a framework in which a key factor of directionality of these interactions is the complexity of the stimulus environment, with a particular emphasis on the ongoing competition among the stimuli within it. Specifically, they suggest that multisensory integration tends to occur more or less preattentively within a scene when there is low competition among the stimuli (Talsma et al., 2010).

2.4 Object Recognition & Object Manifolds

Object recognition is defined as the ability to discriminate objects (identification) or sets of object (categorization) from their background at all possible identity-preserving transformations. Investigating the mechanism of object recognition is a challenging problem because an individual object can produce an infinite number of different images on the retina, due to variations in viewing distance, scale, pose and illumination. Evidence suggests that object recognition is solved in the brain through a cascade of reflexive, feedforward computations that add up to a strong neuronal representation in the inferior temporal cortex (DiCarlo et al., 2012).

Neuronal populations activating to objects appeared under a wide range of conditions form object manifolds. A predominant hypothesis is that along the visual hierarchy, object manifolds are gradually untangled to generate increasingly distinguished object representations, which are linearly decodable (DiCarlo & Cox, 2007). An important tool for understanding higher-level vision is decoding population representations in visual cortical areas of neuronal recordings in mice, rats, and monkeys. This strategy implies that the problem of vision is not one of information content, but of format. It is certain that the activity of retinal ganglion cells contains all the external information that the visual system can use to discriminate objects and that noise in neuronal processing can only decrease the absolute amount of information (Cox, 2014).

2.5 Choosing the second Sensory Modality

Developing behavioral protocols will help to identify the relevant network of the visual cortex involved in object recognition analogous to the ventral stream of primates. Previous studies have shown that mice are capable of invariant object recognition and that lateral visual areas show responses that are more immutable to nuisance transformations (Froudarakis et al., n.d.).

Although sensory perception is traditionally investigated one modality at a time, real world behavior and perception are conducted by the integration of information from multiple sensory sources. Studying object recognition by directly stimulating neurons in the visual cortical areas is a straightforward approach, but is it possible neuronal populations which respond to different

properties of the same object shed some light on object manifolds? In other words, are object manifolds preserved across senses?

To answer this question, we need to train animals to actively associate a visual object with a stimulus from a different sensory modality. We chose olfaction, presumably the most genetically tractable and anatomically compact sensory system. In olfaction, a single sniff is considered a snapshot of the olfactory world and a unit of perception. A previous study has found that mice can make olfactory discriminations of 75% accuracy in 70-90ms after odor inhalation (Resulaj & Rinberg, 2015). Given the fast training and response time in olfactory tasks, we believe that mice have higher chances to associate an odor with a visual object.

2.6 The Purpose of the Study

Despite the significant progress, it's not clear if all the neurons that respond to a certain stimulus are causally responsible for its perception. By forcing animals to link information about objects from different sensory modalities, and using a behavioral task that necessitates mental imagery we hypothesize that the specific neuronal populations that are responsible for the perception of a specific sensory experience will be selectively activated. Nonetheless, a natural environment is by default multimodal and in real life, stimuli rarely demand only one sense.

There is little to no research conducted on object recognition in the animal model of mouse; let alone studying the neuronal and behavioral impact of the association of a visual object to another stimulus modality. The purpose of the study is to design and test behavioral protocols that will ensure the direct association of visual and olfactory stimuli, in order to study neuronal responses in the primary visual cortex (V1) of trained mice. By exposing the mice to the associated odor to the visual object, we can explore the possibility that the manifold of this object is formed in V1 area, using 2-photon imaging, giving us a chance to better identify which subgroup of neurons are responsible for object recognition.

3. Materials & Methods

3.1 Software

3.1.1 Automated behavioral training software: PyMouse

Automation in behavioral experiments is the next necessary step towards creating better and more accurate experimental conditions that will yield undeniable results. New technologies have allowed us to limit animal-experimenter interactions and thus, manual labor, while increasing the number of animals that we can train at once. Multimodal stimuli can be supported to simulate natural environments and accommodate a wide range of experiments, while all data is automatically stored to a database, rendering organization and analysis easier while adhering to the FAIR principles.

All the above were achieved using PyMouse, an open-source state system for automated, high throughput behavioral training implemented in Python and combined with DataJoint toolkit. DataJoint is an open-source framework for programming scientific databases with computational workflows. It provides consistent methods for organizing, populating, computing, and querying data (Yatsenko et al., 2015). All the core components of PyMouse are split into modules that have an abstract defining structure. There are 3 main components: Experiment, Behaviour, Stimulus. Stimulus is responsible for creating and presenting different types of stimuli (visual, olfactory, auditory). Behaviour checks and stores information about the behavior of the animal. Experiment defines the start and stop of the stimulus and the meaning of the animal's behavior at a specific stage of the experiment. In other words, the Experiment component coordinates the Stimulus and Behaviour components.

PyMouse is a state control system, meaning that it provides a clear description of the task by using different states (Figure 3.1). Each Experiment runs in a period called Session for which there are many Trials, consisting of various states (Figure 3.2).

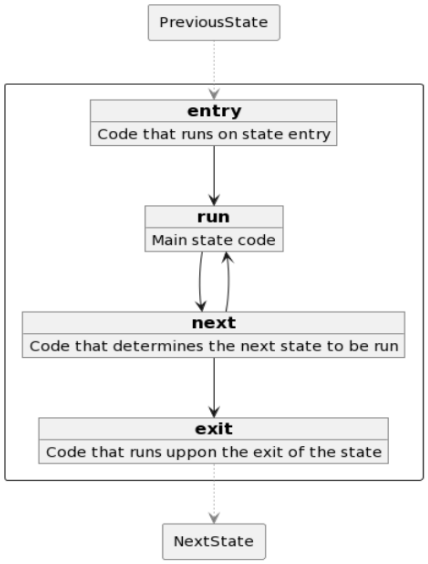


Figure 3.1: A Trial consists of states.

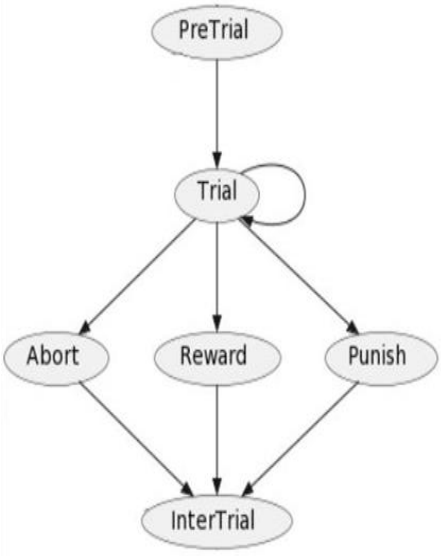


Figure 3.2: A State is described by 4 overridable functions.

3.1.2 Explaining Configuration Files

A Configuration File is a python script in which the experimenter defines the type of Experiment, Behaviour and Stimuli they are using in their experiments. It essentially defines all the session and trial parameters and creates the sets of conditions which characterize the experimental procedure. For instance, it contains parameters about the stimulus presentation (for objects: duration, size, rotation, luminance & for odors: duration, dutycycle of valve, delivery port) and about water consumption (minimum & maximum reward in each session, amount delivered during each correct answer, delivery port).

There are a lot of parameters involved when creating a configuration file. However, for the majority of them, we used the default values described in PyMouse core code. Down below, we present the parameters defining different time periods that needed adjustment and fine tuning throughout the design of the experimental protocols (Table 3.1). Even a small change of milliseconds in these parameters is capable of vastly influencing the animal's performance.

Parameter name	explanation
init_ready	time the mouse needs to spend inside the center port, before the stimulus is presented (initiation)
trial_ready	waiting time between stimulus presentation and response
cue_ready	waiting time inside the center port, during the presentation of cue
abort_duration	time to abort the trial, if the mouse doesn't initiate it by entering its nose inside the center port
punish_duration	waiting time to initiate the next trial, given the mouse has responded incorrectly
cue_duration	time the cue is presented, during which if the mouse responds, the trial gets aborted
response_duration	time to abort the trial if the mouse hasn't responded after cue presentation
intertrial_duration	time until the mouse can initiate the next trial, had it responded or aborted the previous one
delay_duration:	extra time interval between the response and the presentation of reward or punishment
reward_duration:	time the reward is available after the mouse has responded correctly

Table 3.1: Explanation of the parameters defined in configuration file that need fine tuning

3.1.3 Explaining Staircase Selection Trial

PyMouse allows us to run behavioral tasks with various difficulty levels within one session. This essentially means that we can break down the task into steps, as a means of introducing it gradually and effectively to the mice. Especially when having a plethora of conditions which make the task complicated, using staircase selection trial is necessary to identify why and where the animal has trouble to perform. Throughout these experiments, we used this paradigm to split the stimuli combinations and shift the animal's attention to one stimulus at a time and then combining them again within one session.

More specifically, the algorithm was checking the animal's performance (correct/ (correct + wrong) responses) every 20 trials (staircase_window=20). If the performance was above 70% (stair_up = 0.7), the task leveled up and if it was below 55% (stair_down = 0.55), the task leveled down. Difficulty levels below 1 cannot drop to the previous ones.

3.2 Hardware

3.2.1 Home-cage setup using Raspberry Pi boards

The PyMouse software is developed to optimally work on Raspberry Pi boards. The custom-made setup consists of a monitor 7”, connected to a Raspberry Pi 4 board (Rpi). Attached to the Rpi, a control circuit board is regulating the function of solenoid valves, which are responsible for water and air delivery. This board also controls the two lick ports, which are infrared (IR) sensors that detect motion, thus the animal’s licking activity. Each lick port is connected with the output of a valve, and they are located on the right and left side of the setup. In the center, there is a proximity port, which is a photo-interrupter composed of an infrared emitter on one side and a shielded infrared detector on the other. By emitting a beam of infrared light from one upright to the other, the sensor can detect when the animal passes between the uprights, breaking the beam. Behind the proximity port, there is another port responsible for the delivery of odors to the mouse, once the nose of the animal is inside the central proximity port. This port is powered by the Rpi board, and it is connected with the outputs of one or two valves, according to the number of odors used in the experimental protocol.

All the experimental procedures are conducted within the home-cage of the animal. Each propylene cage was adjusted to each setup, by creating holes which accommodate the two lick ports and the proximity center port.

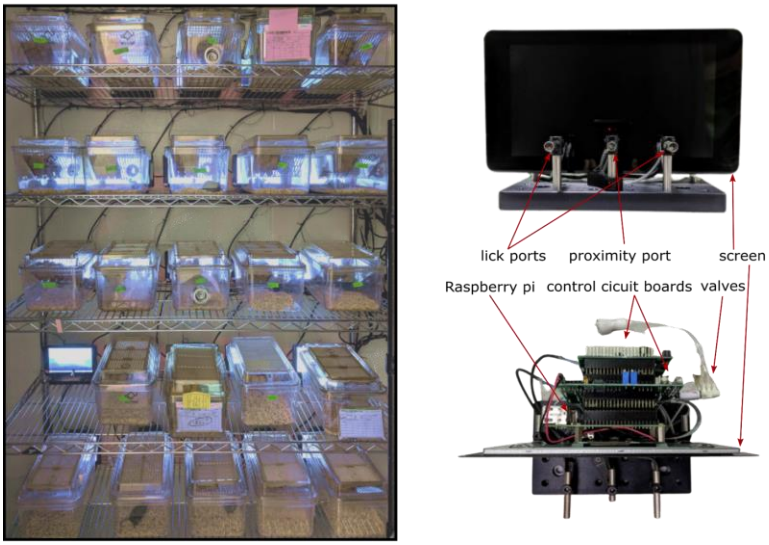


Figure 3.3: Brief depiction of the home-cage setup

3.2.2 Odor-delivery System

Each setup was equipped with an odor delivery system which consisted of two vials (4mL) held by custom-made 3D-printed bases, screwed on the monitor support. Each vial was closed by a black phenolic hole cap, sealed by a PTFE/silicone septa. Penetrating the septa of each vial cap, two needles connected to thin Tygon tubes, were responsible for evoking the odors and were

controlled by one solenoid valve per vial. The input port of the valve was connected to a compressed air distribution piping system fixed at 5PSI pressure, while the output port was connected to one of the needles. The other needle was connected to the odor-delivery port, behind the central photo-interrupter, and would be activated when the mouse would break the beam, introducing him to a 200ms air puff carrying the odor.

3.3 Stimuli

3.3.1 Visual Stimulus

Using Panda3D and Blender, a free open-source framework for 3D visualizations in Python, we were able to generate movies of 3D objects by varying their location, scale, illumination in a continuous manner across time. We varied the following parameters of the objects: X location (translation), magnification (scale), tilt and axial rotation (pose). Previous object discrimination training protocols performed in the lab indicated that mice were able to make the distinction of a cube and a bunny-head shape faster, so these are the chosen shapes (Figure 3.4).

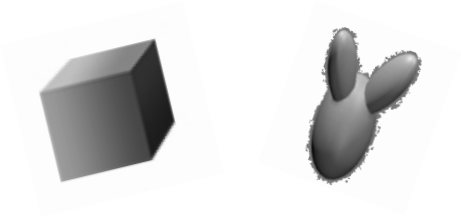


Figure 3.4: 3D objects created with Panda3D

3.3.2 Olfactory Stimulus

Previous behavioral olfactory assays performed in the lab showed that heptanone and benzaldehyde are well received and distinguished by mice. 2-Heptanone ($C_7H_{14}O$), a volatile organic compound, belongs to a class of odorous ketones and it is a colorless, water-like liquid with a banana-like, fruity odor. Benzaldehyde (C_6H_5CHO) is an aromatic aldehyde that possesses a bitter almond, sweet, floral and spice-like odor.

According to previous psychometric olfactory tests, 1μL of each odorant was diluted in 999μL heavy mineral oil, which is an odorless thick liquid that has the capacity to tether the odors, make them last longer and reduce their intensity without interfering with the smell. The total amount in each vial was 1mL and was renewed every week.

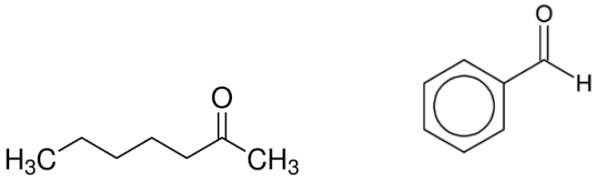


Figure 3.5: Chemical Structure of 2-Heptanone and Benzaldehyde

3.4 Surgical Procedure

For the next three days after the completion of the behavioral training, the animals had ad libitum access to water, to regain their original weight and be able to undergo surgery. During the surgical procedure, a custom-made 3D printed head-post was implanted to each animal and a 4mm cranial window was installed above the right primary visual cortex (V1). The design of the head-post allows sturdy head-fixation of the animal using two screws, which is necessary when conducting 2-photon imaging. In addition, the small well created in the middle of the head-post holds perfectly the liquid lens.

Each subject was administered 0.1mL per 20g weight of a Ketamine/Xylazine mix intraperitoneally, inducing anesthesia for approximately 1-1.5h. During the head-post implantation, the animal was head-fixed using ear bars and placed under the stereotaxic micromanipulator. Next, 0.05mL of local anesthetic Lidocaine was given subcutaneously under the skin of the head, which would be soon removed. After clearing out the skull surface of liquids and tissue, the head-post was attached using Paladur Dental Cement. By that time, a boost injection of 0.05mL per 20gr weight of Ketamine was necessary.

The animal was once again immobilized using the head-post, attached to bars with screws. This facilitates the drilling of the skull, as it offers better stability of the head. With a micro-ruler, 3mm was measured laterally from the midline and 1mm dorsally from lambda, on the right hemisphere. With this point as the center, a circle of 4mm diameter was drilled to the skull and the cranial window was installed using vet glue. Finally, 0.1mL per 30g weight of analgesic Carprofen was administered after surgery, and for the next two days.

3.5 Designing Training Protocols

In this study, we ventured to associate two pairs of stimuli from different modalities. Two visual objects were paired with two odors, as seen in Figure 3.6. We employed two different experimental paradigms to identify the easiest way mice can perceive this association.

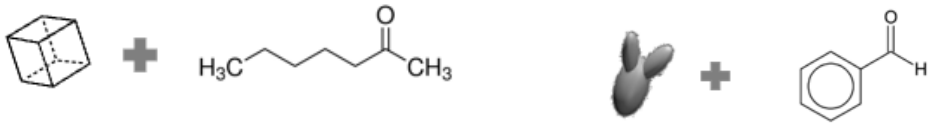


Figure 3.6: Combination of associated stimuli

3.5.1 Go/NoGo Experimental Paradigm

A commonly employed paradigm for studying perceptual decisions is the two-category Go/NoGo task, where the animal performs a response to obtain a reward during a Go – stimulus and needs to withhold the response for the NoGo – stimulus. We selected this training method due to the relative ease with which it can be combined with in vivo 2-photon imaging. Go/NoGo tasks allow the usage of imaging techniques, while the animal is head-fixed and performing the task.

Introducing all four combinations at once to the animals seemed a farfetched endeavor, so we broke down the presentation of conditions into different difficulty levels, using the staircase selection trial. Two C57BL were used for the first set of experiments, following two different approaches, with animal IDs 166 & 167. At the first step of the task (level 0), animal 166 needed to respond when any visual stimulus (cube or bunny head) appeared and withhold when there was absence of stimulus (monitor appeared black). At the second step (level 1), the animal was introduced to the whole task, consisting of 2 correct responses (Go) and 2 incorrect (NoGo), as explained in Figure 3.7. For the animal 167, the task consisted of three difficulty levels. At the first step (level -1), the mouse had to respond when the bunny-head object appeared and wait where there wasn't any stimulus. Next (level 0), both objects appeared, but only the bunny-head was the correct response. Lastly (level 1), the mouse was introduced to the half task, where there were two combinations, characterized by the same odor (Figure 3.8). It was crucial to see if the animal could be trained to at least half the task, before creating a plan for the whole task.

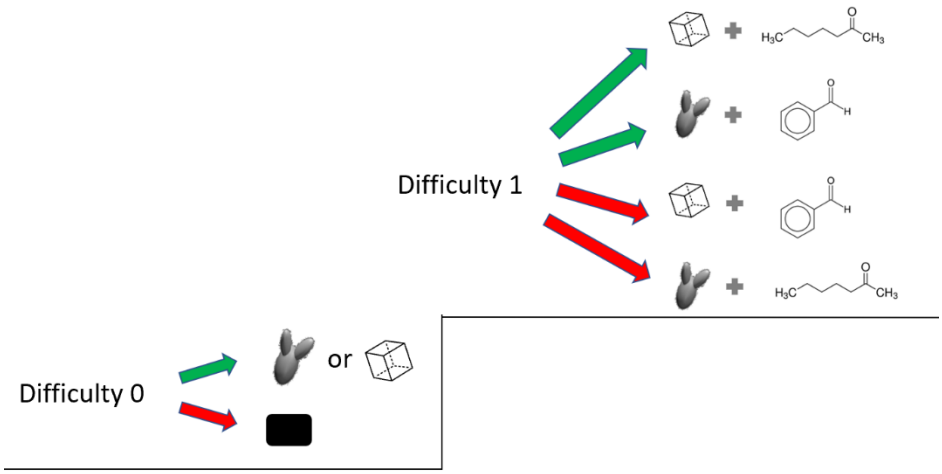


Figure 3.7: preliminary training protocol for animal 166

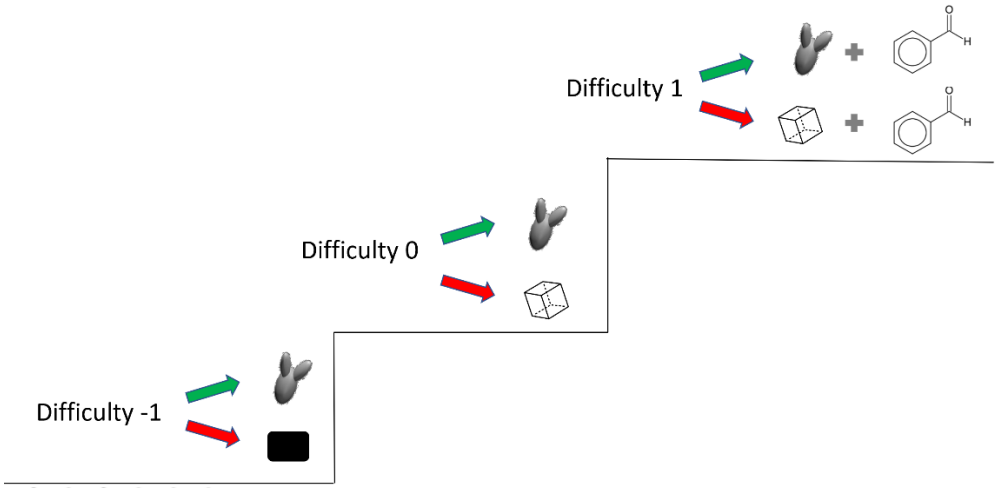


Figure 3.8: preliminary training protocol for animal 167

Calculating the performance of animals in Go/NoGo tasks can be easily accomplished with the help of a confusion matrix. The performance is calculated separately in each difficulty level. More specifically, we use the following terminology to describe all possible combinations of responses and conditions:

- HIT: when the animal responds (Go) to the correct condition
- MISS: when the animal withholds (NoGo) to the correct condition
- FA: when the animal responds (Go) to the incorrect condition
- CR: when the animal withholds (NoGo) to the incorrect condition

Response Type	**Trial Type = correct**	**Trial Type = incorrect**
Go	HIT	False Alarm (FA)
NoGo	MISS	Correct Rejection (CR)

Table 3.2: Confusion matrix for calculation of performance in Go/NoGo tasks

Finally, we adapt the definition of performance (Correct Responses / Incorrect Responses) to the Go/NoGo architecture, and the following formula is formed:

$$P = \frac{HIT + CR}{MISS + FA}$$

3.5.2 Match to Sample Experimental Paradigm (M2S)

In the match-to-sample task, the animal is presented with a sample – stimulus followed by at least two match – stimuli, one of which is the same as the sample, and this is the one that must be chosen by the animal. We selected this experimental paradigm as a simpler alternative to Go/NoGo task that demands not only the animal's attention but also impulsivity control, whereas in M2S tasks, the animal is actively responding at every trial. For this set of experiments, we used GcaMP6 mice (animal IDs: 170, 171, 172, 173, 174), in order to conduct in vivo 2-photon imaging in a passive task after the completion of the training protocol. Additionally, we wanted to identify potential differences in performance from C57BL mice, as these were the first behavioral training of this strain in the lab.

All animals were introduced to the same task, consisting of 3 difficulty levels. At the first step (level -1), the animals were introduced to an object detection task (bunny-head object). The stimulus was presented in the middle of the monitor during the cue period for 200ms, and then it either appeared left or right during the response period for 4s. It goes without saying that the animals were responding correctly when they were choosing the side where the object appeared to get the reward. At the second step (level 0), the animals were introduced to an object discrimination task (target: bunny-head, distractor: cube). This time, during the response period the two objects

appeared left and right (not on a fixed side), and the animals had to follow the target-stimulus and avoid the distractor. At the last difficult level, we introduced the odor (benzaldehyde) that was paired with the target stimulus (bunny-head), during the cue period. The odor would be presented for 200ms, while the animals' nose was inside the proximity center port.

In M2S tasks, the performance is calculated by the simple formula of (Correct Responses / Incorrect Responses). It is calculated separately in each difficulty level. Testing these training protocols led to changes that will be thoroughly discussed and explained in the Results section. Obviously, the association of stimuli is ensured only when the animals are performing efficiently the whole task, consisting of all four conditions.

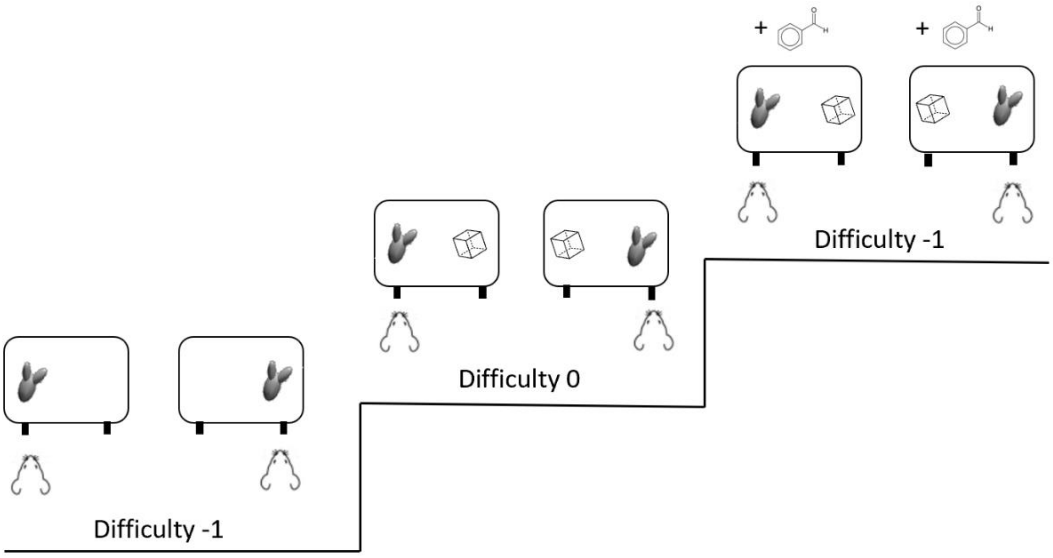


Figure 3.9: M2S training protocol

3.5.3 Two Alternative Forced Choice Experimental Paradigm (2AFC)

A simplified version for the association task was created using the two-alternative-forced-choice experimental paradigm, as each stimulus appears consistently at the same side of the monitor. As shown in Figure 3.10, the lick ports on each side were attributed to one specific visual object (left for bunny-head and right for cube). At the first step (level 0), the stimuli appeared on the congruent side of the monitor in order to help the animals create the desired association. At the next step (level 1), both stimuli appeared in the center, so the animal had to go left when the bunny-head appeared, or right when the cube appeared in order to receive the reward. The reason why we didn't introduce any odorant stimuli to the animals in this task yet is because they would ignore the visual stimuli and perform based on the olfactory stimuli, which they learn in only 2 sessions.

Animals 170 & 171 were introduced to this training protocol after failing at the M2S association task. In 2AFC tasks, the performance is calculated by the simple formula of (Correct Responses / Incorrect Responses) and it is calculated separately in each difficulty level.

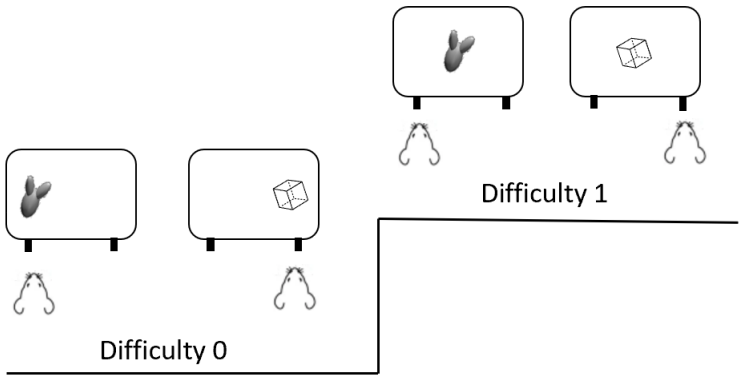


Figure 3.10: 2AFC training protocol (2)

3.6 Animals

3.6.1 Housing & Care

Seven mice (3-4 months old, 2 C57BL/6J mice, both male & 5 Thy1-GcaMP6sGP4.3 mice, 3 female and 2 male) were housed in a temperature-controlled (~23ºC) and humidity-controlled (~55%) facility on a 12/12h light/dark cycle; zeitgeber time (ZT)0 = 8 A.M. All procedures were approved by the Research Ethics Committee of University of Crete and followed the European Parliament protocols on the protection of animals used for scientific purposes. Animals were allowed to acclimate for at least one week after receival, with ad libitum access to food and water before water regulation was initiated (daily water intake was approximately 1.2mL).

3.6.2 Mice Strains

The strain C57BL/6J is the most widely used inbred strain and the first to have its genome sequenced. We used two mice of this strain to conduct the preliminary testing of the developing experimental procedures. Once we gained the first data, we trained GcaMP6 mice, aiming to perform 2-photon imaging, after the conclusion of the training. These transgenic mice express the green fluorescent calcium indicator GcaMP6s in subsets of excitatory pyramidal neurons in the brain. The transgene contains a mouse *Thy1* promoter (GcaMP6s), woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and bovine growth hormone (bGH) polyadenylation sequence. The GcaMP6s indicator is an ultrasensitive detector of single neuronal action potentials and has slow response kinetics (Chen et al., 2013). Consequently, these mice can be used for imaging-based monitoring of neuronal activity in individual neurons in the brain, while stable expression levels in the cortex are sufficient for *in vivo* imaging.

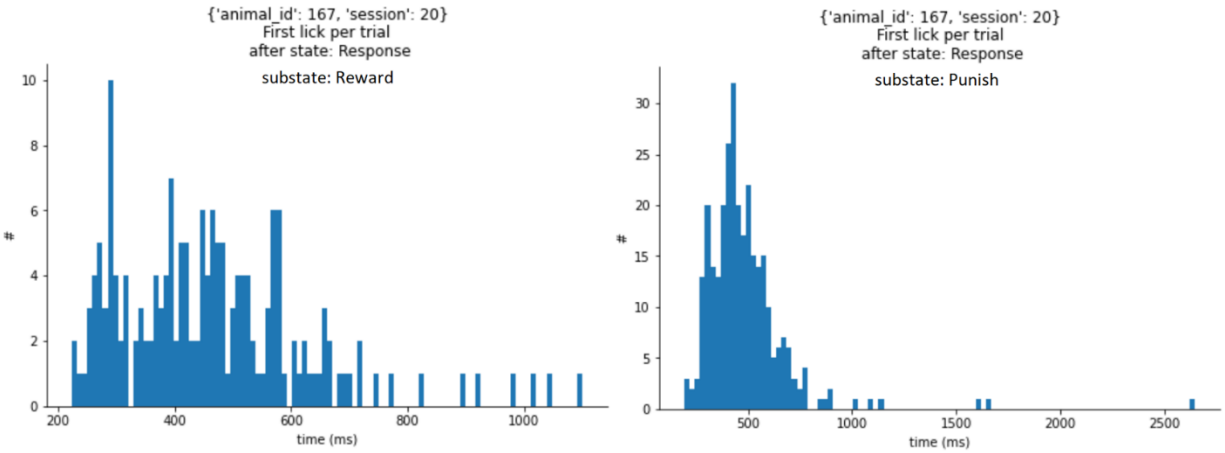
4. Results

4.1 Insight on Go/NoGo tasks

Due to experimental issues, the results for animal 166 are inconclusive. The majority of the sessions could not be taken into consideration as they were hardware malfunctions.

Meanwhile, animal 167 showed us that small changes in the time parameters of the experiment can make a huge difference in the animal's performance. Through trial and error, we identified some common oversights that highly affect the outcome of the behavioral experiments.

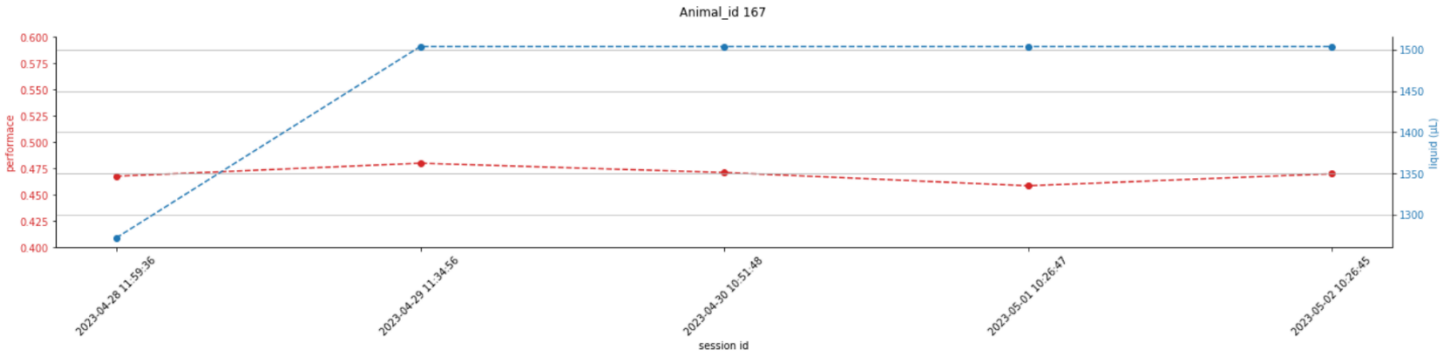
a) The response time plays a significant role in Go/NoGo tasks: if it is wrongfully long it can be unnecessarily hard for the mouse to perform correct rejections. The mouse should consciously reject the wrong combination, but not wait too much to do so, as waiting is the form of punishment we use. Initially, the response time was set to be 3ms, but after the first 20 sessions, it was found that the average response time for mice is naturally between 200-1000ms. Consequently, we set the response time to be 1.5ms. In the plot below we showcase the response time of mouse 167 during Reward and Punish period in a typical session. As we see, during the Punish period, some responses were outside the typical response time range that could be correct rejections.



Plot 4.1a: Response time in a typical session

b) The total amount of water the animals consume should be relative to their performance, while making sure they always drink the minimum quantity. However, the duration of the session should be finely tuned with the reward amount, so the animals don't drink excessive water when they are not performing well. This also depends on the number of aborted trials and the punishment duration. We found that with a session duration of 45mins, reward amount of 6μL, punishment duration of 8s and minimal aborts, the amount of water the animals consume is consistent with their performance. In the plot bellow, we showcase a distinctive example of the early sessions of animal 167, which was performing at chance level for several days, while drinking the maximum amount of

water. Naturally, the animal didn't have to learn the task, or change strategy once he drunk the water.



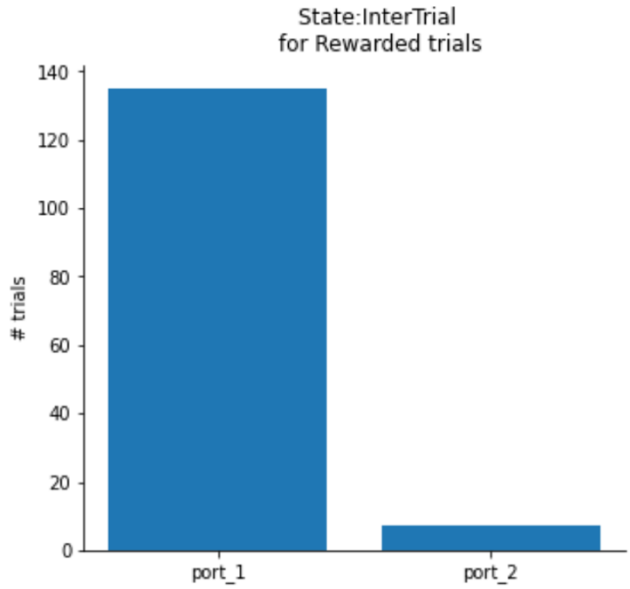
Plot 4.1b: The total amount of water should be relative to the performance

c) When using a staircase paradigm, it is important to reevaluate the steps often. Once the animal gets acquainted with the first difficulty levels, soon they can be omitted so the animal has more trials of the main task.
d) Overrepresenting the NoGo condition may help animals learn the task easier. Moreover, having an antibias system is a necessary means to control the water intake while ensuring the animals are actively learning the task and not performing at chance levels.

4.2 Insight on Match-to-Sample tasks

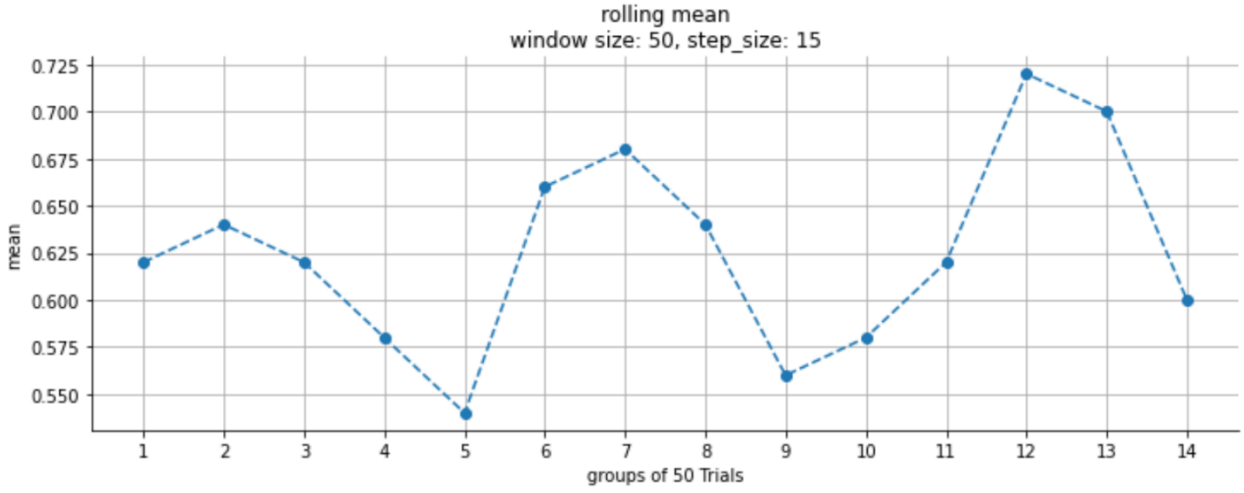
Three out of five Thy1-GCaMP6 mice had difficulties performing even at the simplified steps of the task (object detection), resulting to a decrease of our cohort to two mice. We could not get any valuable information from animals 172, 173, 174 regarding the behavioral settings, however we gained some insight from animals 170, 171.

a) During the first days of water deprivation through the Rpi set up, mice instantly develop a bias to the port they initially chose to drink from randomly. A better strategy would be to force the animals to drink from both lick ports during the free water task, by allowing them to drink only from one port every 15 trials. We show the preference to each port of animal 170, session 22 in an object detection task as a typical example. During the free water task, the animal was receiving the water from port 1, which was also its preference during the first days of the detection task. It was hard to render the animals unbiased again, but we achieved so by running a few sessions of free water delivered only from port 2.



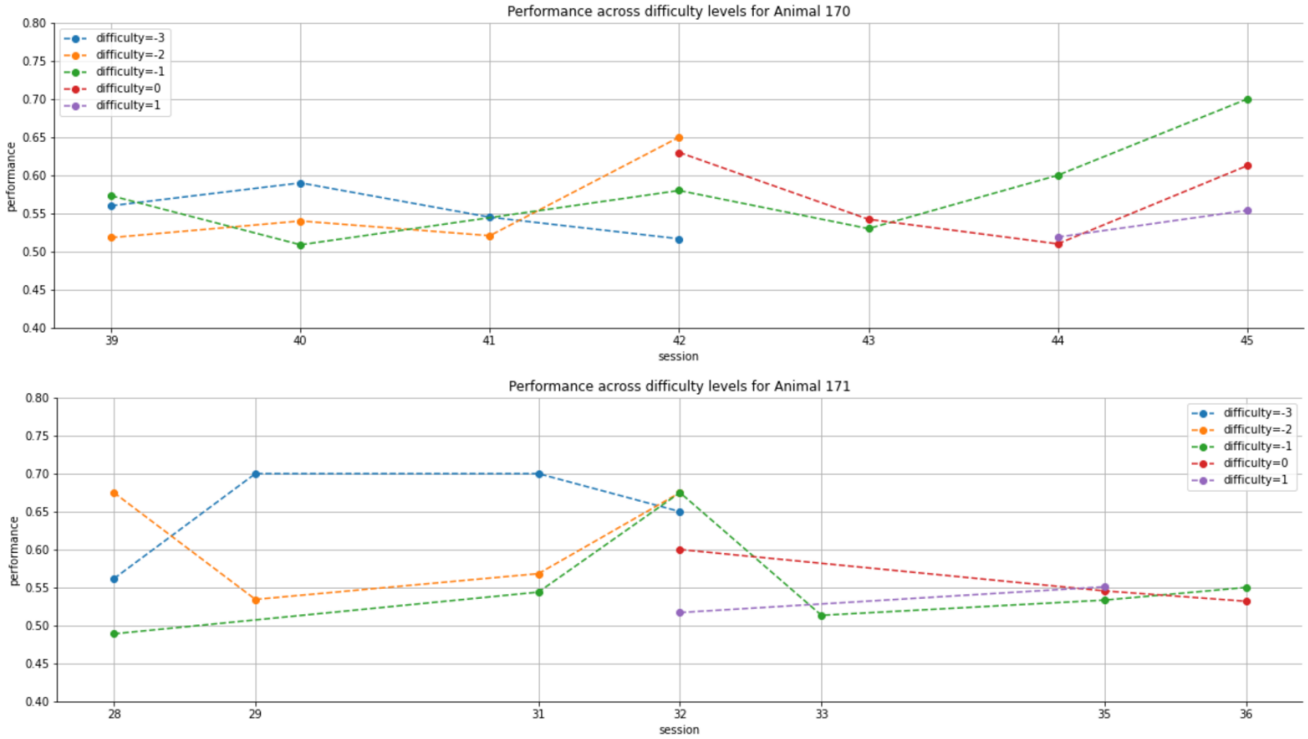
Plot 4.2a: Bias towards a single port during free water tasks

b) The performance of Thy1-GCaMP6 mice fluctuates a lot within one session, which may be related to a temporary satisfaction of thirst. We found that it is challenging to keep the animals motivated throughout the session, even if they have not yet consumed all the amount of water they could. We show a characteristic example of this fluctuation from animal 170, session 76. The average performance in that session was 0.61. In this case, the performance peaks both at the beginning, middle and end of the session. On other days, the performance peaked only in one or two stages of the session.



Plot 4.2b: Fluctuation of performance within a session

c) During object discrimination tasks, introducing the distractor from smaller to equal magnification levels to the target object didn't seem to have helped the animals significantly. We did so, using staircase selection trial, by gradually magnifying the distractor in each difficulty level, until it was the same size as the target object (0.5). We initially used 5 difficulty levels (0.1, 0.2, 0.3, 0.4, 0.5) and as the animals performed better, the steps decreased (0.2, 0.35, 0.5). Technically, at small magnifications, the task becomes an object detection task, thus the animals reach a performance of 0.7 in a few sessions. However, as the distractor becomes comparable to the target in size, mice performed at chance level once again.



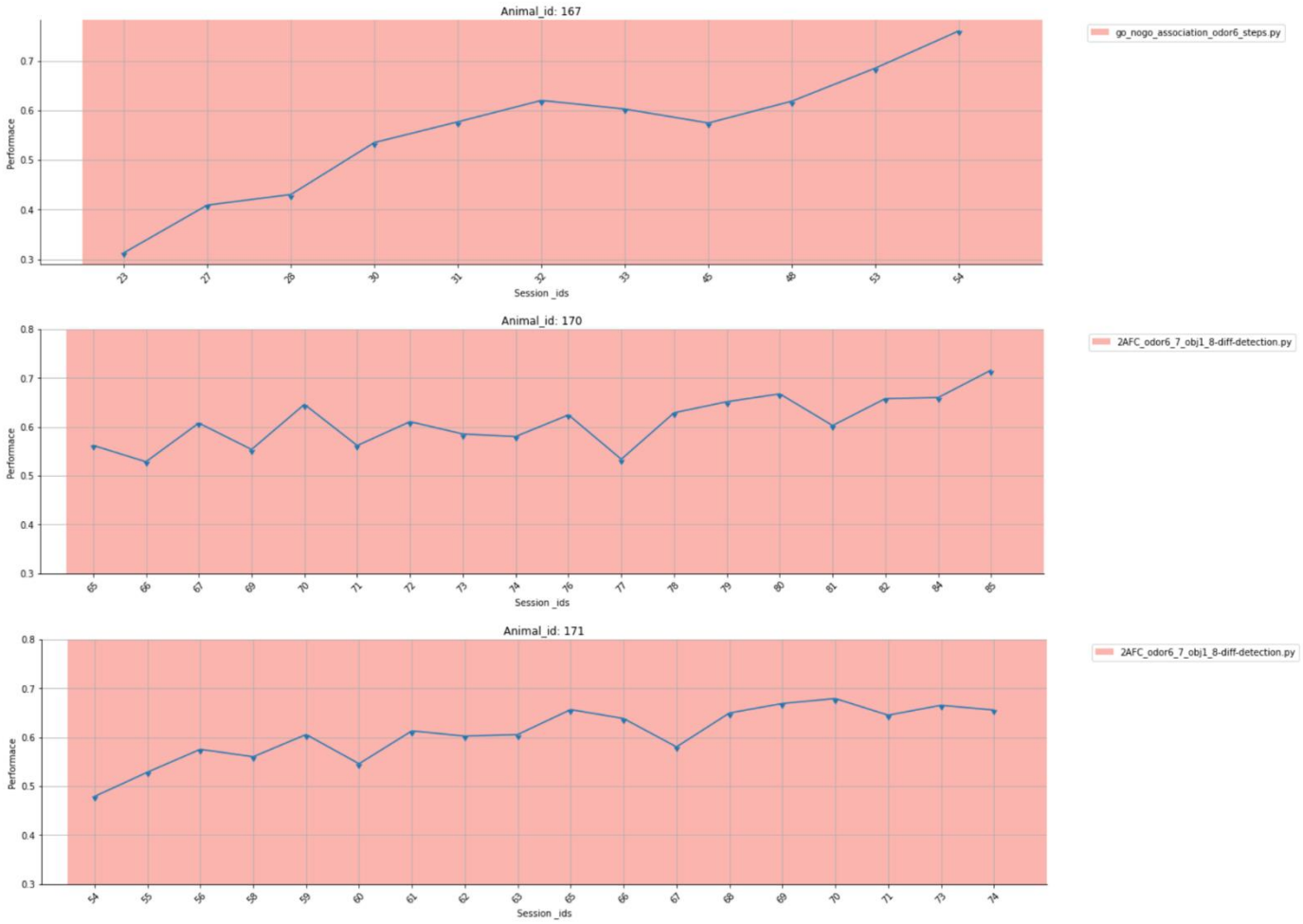
Plot 4.2c: Performance across difficulties

d) When employing a multimodal stimulus, we need to prove that the animals use both modalities in order to complete the task. To do so, we should perform some preliminary recordings mapping the responses to each modality and then to the task as a whole.

4.3 Thy1-GCaMP6 mice learn slower than C57BL/6J mice

A trend that was quickly apparent from our small cohort was that Thy1-GCaMP6 mice needed more days to perform adequately in object discrimination tasks. While both strains took 6-7 days to reach 70% of performance in object detection tasks, in discrimination tasks there were great differences. The C57BL/6J mouse (167) reached 70% of performance in about 2 weeks (11 days), while Thy1-GCaMP6 mice (170,171) in about 3 weeks (18 days). A huge issue regarding the behavioral training of Thy1-GCaMP6 mice was that they didn't increase continuously their performance as they learnt the task across days. There were many inconsistencies in their performance from day to day that could not be accounted for by any external factors. Some days they reached a 65% performance and the next nearly above chance levels. This was the main reason why Thy1-GCaMP6 mice needed considerably more days to perform object discrimination tasks than C57BL/6J.

Note that animal 167 has been trained in the Go/NoGo discrimination task, as explained in Figure 3.8, while animals 170 & 171 have been trained in the 2AFC (2) discrimination task, as explained in Figure 3.10.

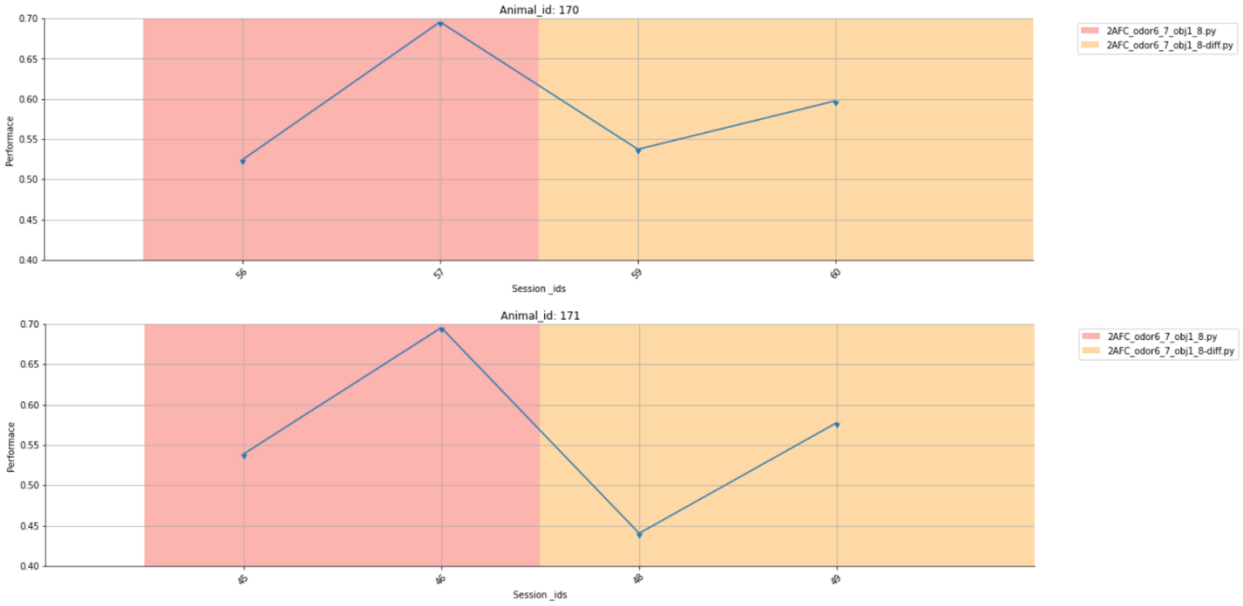


Plot 4.3: Thy1-GCaMP6 mice learn slower than C57BL/6J mice

4.4 Mice tend to get fixated on the olfactory rule

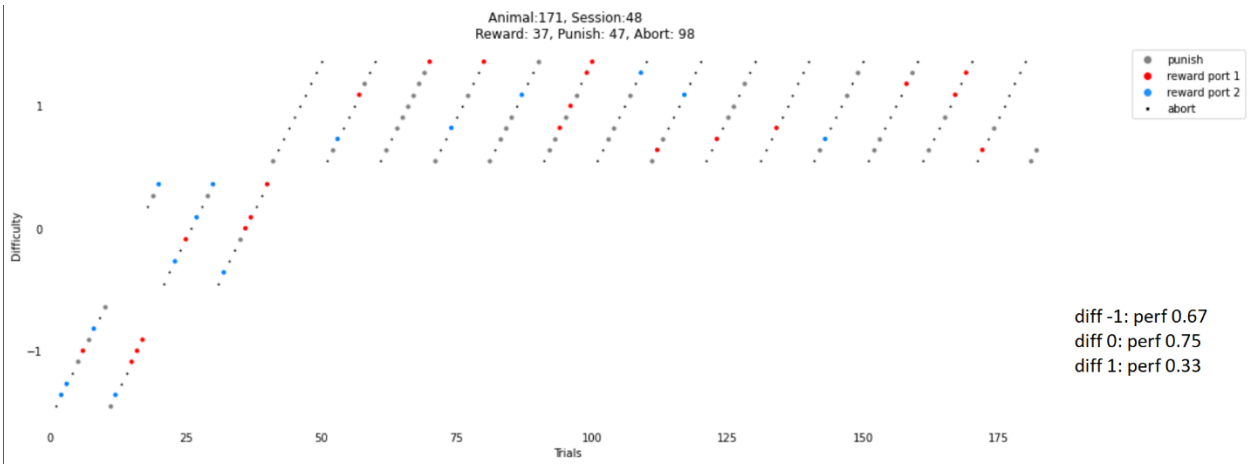
Mice used mainly the olfactory stimulus to navigate the tasks, if it was possible. Especially during tasks that we combined odorants with visual objects, it was apparent when they were totally ignoring the visual information. If the discrimination was achieved in the first sessions, they only utilized the olfactory stimulus to make decisions. It goes without saying that we quickly refrained from using behavioral training protocols in which this kind of maneuvers were possible. However, it seemed hard to establish the association while simultaneously ensuring that mice utilize both stimuli to make decisions.

Each combination of odorant-visual stimulus was attributed to each lick port. For sessions 56, 57 & 45,46 the two mice were exposed to the complete combinations, and both reached a performance of 70% in just two days, indicating that they only paired the different odors with the two ports, ignoring the visual stimulus. For sessions 59, 60 & 48, 49 we incorporated two difficulty steps to the task. The first was the same as the task before (valve dutycycle: 100%). In the second one, the odors were a lot less intense (dutycycle: 25%) and in the third one, there was only visual stimulus. It is important to note that plots show the combined performance of the three difficulty levels in an object detection task for each mouse. We can see that the performance is dramatically reduced in the last difficulty level, whereas the intensity of the odor doesn't play a significant role; as long as it can detected, mice get fixated on it.



Plot 4.4a: Mice tend to get fixated on the olfactory rule

As a typical example, we show the lick plot of animal 171, session 48, accompanied with the performances at each difficulty level. Another striking observation was that as soon as odors ceased to be presented, animals were aborting many trials, by not staying long enough in the center port. Apparently, odors also acted as an indicator of the time the animals should wait inside the center port.



Plot 4.4b: As odors cease to be presented, performance drops dramatically

5. Discussion

Understanding how sensory information is processed requires a variety of approaches that cover multiple levels of investigation, from genes to neurons to behavior. There are certainly limits on behavioral performance that constrain the possible neural mechanisms responsible for specific computations.

As mice are non-foveal, they may rely more on head movements rather than eye movements to view certain areas of the visual space. As a result, they are not the ideal model to study high complexity object recognition tasks. It goes without saying that behavioral assays should be adapted to tasks mice can accomplish, concerning both stimulus parameter range and the way of reporting responses.

The extent of visual perception in mice that depends on the cortex is still unclear. In general, it is of utmost importance to ensure that a particular task relies on the brain region being studied, even if it has been proved in other species. That being said, it is preferable to conduct a series of preliminary recordings on trained mice in a simple 2AFC object discrimination task, before embarking on a more complex association of stimuli.

5.1 Olfaction may not be the best modality to create cross-modal associations in mice

Rodents are olfactory specialists and are adept at using odors to learn contingencies quickly and effectively. A recent study showed that mice can rapidly learn to categorize multiple odors into groups that are either rewarded or unrewarded. They conducted single-cell recordings in male and female C57BL/6J mice while engaging in behavioral tasks and revealed that an explicit representation of reward categories emerges within the olfactory tubercle (OT) within minutes of learning a new odor-reward association. This clear representation of reward categories in the OT is apparent within the first sniff (lasting 50–100 ms) of an odor in each trial and precedes the execution of motor actions (Millman & Murthy, 2020). Interestingly, another study showed that the olfactory tubercule appears to function as a point of direct multimodal convergence during the initial stage of odor processing, potentially acting as a location for perceptual interactions between odors and sounds (Wesson & Wilson, 2010).

In our sets of behavioral experiments, we use the same odors for both rewarded and unrewarded combinations of stimuli. Olfactory stimuli seem to be strongly perceived by rodents, naturally being the behavioral drive during perception. In this way, they cover any associated stimulus and thus, mice innately pay close attention only to the predominant stimuli, odors.

5.2 The question of Bidirectionality & Multisensory Integration

In this study, we have focused on olfactory influences on visual scene analysis and only within the primary visual cortex. A complete approach would be to also examine if the visual stimulus affects olfactory judgements in the same cross-modal object. Are these behavioral predictions bidirectional across modalities?

Being in a position to correctly interpret results from a multimodal association of stimuli requires not only a very deep understanding of neural circuits interactions, but also being able to distinguish the cause and effect in these processes. In this regard, there are a lot more questions to be addressed. For instance, recent emphasis on multisensory attentional processing highlights the importance of stimulus competition in uncovering these interactions. In the case of individual olfactory or visual event sequences for mice, odors are highly salient. How do the processes of bottom-up sensory integration and top-down attention interact, both in terms of behavior and at cellular level?

This study failed to establish a cross-modal association of olfactory and visual stimuli using traditional behavioral paradigms (Go/NoGo, M2S), consequently more questions arise. Is the level of attention needed for multisensory integration affected by the complexity of the perceptual task, or is it solely determined by the complexity of the stimulus input, regardless of the task?

5.3 Using Thy1-GCaMP6 mice in object recognition tasks is challenging

There is insufficient research regarding the learning impairments of transgenic Thy1-GCaMP6 mice. Only two out of six Thy1-GCaMP6 mice were able to learn a simple object detection task, and later an object discrimination task. We acknowledge that our cohort is small, but further experiments should be conducted to determine if this strain is as capable of object recognition as C57BL/6J mice.

In retrospect, a more promising strategy would be to perform intracranial injection of viral vectors engineered to express a fluorescent protein to C57BL/6J mice and train this strain at object recognition tasks. There is an urgent need to shed some light on the characteristics of the developing transgenic lines, in order to rule out potential impediments in future studies. We must not forget that research also makes progress through negative results, which often have great practical value.

6. References

Chen, T.-W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., Schreiter, E. R., Kerr, R. A., Orger, M. B., Jayaraman, V., Looger, L. L., Svoboda, K., & Kim, D. S. (2013). *Ultra-sensitive fluorescent proteins for imaging neuronal activity* (Vol. 499, Issue 7458). http://www.nature.com/authors/editorial_policies/license.html#terms

Cox, D. D. (2014). Do we understand high-level vision? In *Current Opinion in Neurobiology* (Vol. 25, pp. 187–193). https://doi.org/10.1016/j.conb.2014.01.016

DiCarlo, J. J., & Cox, D. D. (2007). Untangling invariant object recognition. *Trends in Cognitive Sciences*, *11*(8), 333–341. https://doi.org/10.1016/j.tics.2007.06.010

DiCarlo, J. J., Zoccolan, D., & Rust, N. C. (2012). How does the brain solve visual object recognition? *Neuron*, *73*(3), 415. https://doi.org/10.1016/J.NEURON.2012.01.010

Froudarakis, E., Cohen, U., Diamantaki, M., Walker, E. Y., Reimer, J., Berens, P., Sompolinsky, H., & Tolias, A. S. (n.d.). *Object manifold geometry across the mouse cortical visual hierarchy*. https://doi.org/10.1101/2020.08.20.258798

Huberman, A. D., & Niell, C. M. (2011). What can mice tell us about how vision works? *Trends Neurosci*, *34*(9), 464–473. https://doi.org/10.1016/j.tins.2011.07.002

Li, D., Wang, G., Xie, H., Hu, Y., Guan, J. S., & Hilgetag, C. C. (2019). Multimodal Memory Components and Their Long-Term Dynamics Identified in Cortical Layers II/III but Not Layer V. *Frontiers in Integrative Neuroscience*, *13*. https://doi.org/10.3389/fnint.2019.00054

Meredith, M. A. (2002). On the neuronal basis for multisensory convergence: A brief overview. *Cognitive Brain Research*, *14*(1), 31–40. https://doi.org/10.1016/S0926-6410(02)00059-9

Millman, D. J., & Murthy, V. N. (2020). Rapid Learning of Odor–Value Association in the Olfactory Striatum. *Journal of Neuroscience*, *40*(22), 4335–4347. https://doi.org/10.1523/JNEUROSCI.2604-19.2020

Naselaris, T., Olman, C. A., Stansbury, D. E., Ugurbil, K., & Gallant, J. L. (2015). A voxel-wise encoding model for early visual areas decodes mental images of remembered scenes. *NeuroImage*, *105*, 215. https://doi.org/10.1016/J.NEUROIMAGE.2014.10.018

Pearson, J. (2014). New Directions in Mental-Imagery Research. *Https://Doi.Org/10.1177/0963721414532287*, *23*(3), 178–183. https://doi.org/10.1177/0963721414532287

Pearson, J., Naselaris, T., Holmes, E. A., & Kosslyn, S. M. (2015). Mental Imagery: Functional Mechanisms and Clinical Applications. *Trends in Cognitive Sciences*, *19*(10), 590. https://doi.org/10.1016/J.TICS.2015.08.003

Rao, R. P. N., & Ballard, D. H. (1999). Predictive coding in the visual cortex: a functional interpretation of some extra-classical receptive-field effects. *Nature Neuroscience*, *2*(1), 79–87. https://doi.org/10.1038/4580

Resulaj, A., & Rinberg, D. (2015). Novel Behavioral Paradigm Reveals Lower Temporal Limits on Mouse Olfactory Decisions. *The Journal of Neuroscience*, *35*(33), 11667. https://doi.org/10.1523/JNEUROSCI.4693-14.2015

Talsma, D., Senkowski, D., Soto-Faraco, S., & Woldorff, M. G. (2010). The multifaceted interplay between attention and multisensory integration. *Trends in Cognitive Sciences*, *14*(9), 400–410. https://doi.org/10.1016/J.TICS.2010.06.008

The Case for Mental Imagery - Stephen M. Kosslyn, William L. Thompson, Giorgio Ganis - Βιβλία Google. (n.d.). Retrieved October 11, 2023, from https://books.google.gr/books?hl=el&lr=&id=F45JCAAAQBAJ&oi=fnd&pg=PR7&dq=Kosslyn+S.M.+The+Case+for+Mental+Imagery+2006+Oxford+University+Press+&ots=v1AXjwDR6Y&sig=MCPZ6_m4g2N1YSxutz-qbME48Vo&redir_esc=y#v=onepage&q=Kosslyn%20S.M.%20The%20Case%20for%20Mental%20Imagery%202006%20Oxford%20University%20Press&f=false

Van der Burg, E., Olivers, C. N. L., Bronkhorst, A. W., & Theeuwes, J. (2008). Pip and pop: nonspatial auditory signals improve spatial visual search. *Journal of Experimental Psychology. Human Perception and Performance*, *34*(5), 1053–1065. https://doi.org/10.1037/0096-1523.34.5.1053

Van Ee, R., Van Boxtel, J. J. A., Parker, A. L., & Alais, D. (2009). Multisensory Congruency as a Mechanism for Attentional Control over Perceptual Selection. *The Journal of Neuroscience*, *29*(37), 11641. https://doi.org/10.1523/JNEUROSCI.0873-09.2009

Wang, Q., Gao, E., & Burkhalter, A. (2011). Gateways of ventral and dorsal streams in mouse visual cortex. *Journal of Neuroscience*, *31*(5), 1905–1918. https://doi.org/10.1523/JNEUROSCI.3488-10.2011

Wesson, D. W., & Wilson, D. A. (2010). Smelling sounds: olfactory-auditory sensory convergence in the olfactory tubercle. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *30*(8), 3013–3021. https://doi.org/10.1523/JNEUROSCI.6003-09.2010

Yatsenko, D., Reimer, J., Ecker, A. S., Walker, E. Y., Sinz, F., Berens, P., Hoenselaar, A., Cotton, R. J., Siapas, A. S., & Tolias, A. S. (2015). DataJoint: managing big scientific data using MATLAB or Python. *BioRxiv*, 031658. https://doi.org/10.1101/031658