The amygdala in value-guided decision making

by

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Submitted to the Department of Brain and Cognitive Sciences in partial fulfillment of the requirements for the degree of

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Abstract
The amygdala is a structure well known for its role in fear and reward learning, but how these mechanisms are used for decision-making is not well understood. Decision-making involves the rapid updating of cue associations as well as the encoding of a value currency, both processes in which the amygdala has been implicated. In this thesis I develop a strategy to study value-guided decision making in rodents using an olfactory binary choice task. Using a logistic regression model, I show that the value of expected rewards is a strong influence on choice, and can bias perceptual decisions. In addition, I show that decisions are influenced by events in the near past, and a specific bias towards correct choices in the near past can be detected using this analysis. Using genetic targeting of a sub-population of amygdala neurons, I show that this population is required for the rapid learning of an olfactory decision making task. Using in-vivo calcium imaging of this population I show that these neurons are active during the inter-trial interval and modulated by choice history, suggesting a mechanism by which choice history can influence current decisions.

Thesis Supervisor: Susumu Tonegawa

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I dedicate this thesis to my husband Olivier Girault.
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Chapter 1

Introduction

The amygdala is a complex almond-shaped structure that has been evolutionarily conserved across the animal kingdom. The complexity of the structure arises from both the extensive interconnectivity with other brain structures, as well as the diversity of cell types organized into different nuclei within the structure. Not surprisingly, a multitude of processes have been ascribed to its function. One of the best studied processes is the associative learning of threat-inducing stimuli. The best example of this is Pavlovian fear conditioning, in which a neutral sensory stimulus (tone, odor, etc.) is paired with an aversive stimulus (air puff, electrical foot shock). This creates a strong and lasting association, which can be measured by the animal's behavior (freezing). For this particular task, the contributions of various amygdala nuclei have been characterized. Although this is the most well-studied aspect of amygdala function, various avenues of research have also shown that the amygdala is involved in learning about rewarding stimuli. For example, it is known to be involved in tasks such as operant conditioning and reinforcer devaluation. These tasks involve both...
associating an action with a particular reward, as well as tracking the value of the reward in order to change a course of actions.

The involvement in these seemingly opposite processes has led to the hypothesis that the main function of this structure is to encode the value of different outcomes, in essence associating an outcome with a value from a scale ranging from very negative to very positive. This associative information can then be transmitted to produce the appropriate behavior. Electrophysiological studies further corroborate this hypothesis by showing that in fact neurons in the amygdala encode the value of an outcome when it occurs. Subsequently after an animal has learned to associate a predictive cue with an outcome, these neurons will respond to predictive cues in a manner consistent with the value of the expected reward.

The encoding of a value parameter is critical for all aspects of decision-making. It can be as simple as a decision to approach versus retreat from a given environment, or as complex as deciding which move to play next in a game of chess. In fact, many learning algorithms use a value parameter in order to solve very complex problems. In this thesis I aim to study how value signals evolve as an animal learns, and how these signals might be used to determine behavior.
1.1 - Thesis Contributions

This thesis looks at the role of the amygdala in value-guided decision-making.

- I describe the development of an olfactory decision-making task for use with mice that lends itself to the study of value-guided choices
- I describe a strategy for analyzing decision-making data dynamically
- I show that a subset of amygdala neurons that are RSPO2+ are required for integrating choice-related information
- I show that the activity of RSPO2+ neurons is modulated by prior outcomes, consistent with their role in integrating prior outcomes into current choices.

1.2 - Thesis Organization

Chapter 2 - Prior Outcomes and Expected Outcomes Produce a Bias in Current Decisions.

This chapter describes the development of a sensory decision-making task for mice. It describes the training of mice using cues of odor mixtures that predict rewards of varying sizes. By mixing odors to varying degrees I was able to create cues of varying difficulty. This allowed us to look at how animals behave when the predictive value of a cue is uncertain. By creating mixtures of odors that predicted rewards of different values, I was able to show that expected reward values can bias an animal’s decision.
when the predictive value of a cue is uncertain. I also show that decisions are also biased by outcomes that occurred in the recent past, implying a mechanism for behavioral updating. I then go on to use this task to study the role the amygdala might play in producing these two types of biases.

Chapter 3. RsPo2+ Neurons are Required for Integrating Prior Outcomes into Current Decisions.

In this chapter I show the characterization of a mouse transgenic line that expresses CRE recombinase under the promoter elements of the RsPo2 gene. I show that CRE expression is confined to the basal nucleus of the basolateral amygdala complex. I then show that these neurons can be specifically ablated using CRE-responsive virus that expresses an activated caspase enzyme. Using this strategy to ablate RsPo2+ neurons, I show that these neurons are required for efficient learning of a value-guided decision-making task. I specifically show that this learning deficit is specifically due to a deficit in integrating prior outcomes into current decisions, indicating that RsPo2+ neurons may be responsible for integrating information across trials.

Chapter 4. RsPo2+ Neurons Encode Prior Outcomes Among Other Decision Variables.
In this chapter I perform live imaging of putative Rspo2+ neurons in behaving animals using a virally-encoded GCaMP protein in combination with a miniature endoscope. By labeling Rspo2+ neurons with GCaMP in vivo, I was able to capture the dynamic encoding of putative Rspo2+ cells while the animal learns a value-guided decision-making task. I show that putative Rspo2+ neurons are inhibited by reward-predictive cues, and are activated in the inter-trial interval (post-reward period), consistent with its recently-described role in fear learning. In addition, the activity in the inter-trial interval is modulated by prior outcomes, suggesting a mechanism by which these putative neurons may be used to integrate information across trials. This chapter also serves as a general characterization of the neural correlates in the amygdala during decision-making. I use a general neuronal promoter to express GCaMP in all amygdala neurons. Using this procedure, I show that putative amygdala neurons encode both correct and incorrect outcomes.
Chapter 2

Prior Outcomes and Expected Outcomes Produce a Bias in Current Decisions.

This chapter describes the development of an odor categorization task in mice that allows for the distinction of various influences on animal choice behavior. A two-alternative forced choice task is developed in which odors are used to indicate the direction, left or right, which will yield a reward. Animals learned by trial and error the associations between each odor identity and the action that yielded a reward. By using several odors, each predicting a reward of a different size, I was able to study the influence of reward value on any given choice. In addition, by presenting the animal with more perceptually difficult choices – by mixing the odors together – I was able to measure the influence of reward value in situations where perceptual uncertainty exists. Using a regression model of the behavior data, I find that decisions are biased by the expected value of future rewards, and also by the history of choices made by the animal. These two effects had varying effects, depending on the difficulty of the choice.
2.1. Introduction

Decision-making involves using sensory cues to predict the outcomes of all possible actions that can be taken. Unlike a reflex, such as your knee reflex, in which a stimulus elicits a stereotyped response, a decision-making system is capable of evaluating the potential consequences of any actions and selecting the action that leads to the most desired result. This inherently requires a mechanism to assign value to different actions and outcomes, and it is becoming increasingly clear that the amygdala plays a large role in this evaluation.\(^1\) Animal studies using lesions of the amygdala have shown that the amygdala is required for tracking changes in reward value.\(^2\)\(^3\)\(^4\) Recording studies using electrophysiology have shown that the amygdala encodes the value of future outcomes, and that this encoding changes dynamically as the animal learns.\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)

Animal decision-making is often characterized by an action that is taken in response to some external stimuli in the environment. It is often categorized as being either perceptual decision-making or value-based decision making.\(^10\)\(^11\) Perceptual decision-making, as the name suggests is characterized by an ability to distinguish the different features of a stimuli, such as deciding if one is at a green light or a red light, or distinguishing one’s car among a set of similar cars. In these situations, it is the sensory features of the stimulus that determines what action one takes.
characteristic feature of perceptual decision-making is that there is a correct choice; the light is either green or not green, the car is either your car or it is not. In value-based decision-making it is not the sensory features, but the underlying value that determines the choice. A simple example of this is deciding between an apple and an orange, where they are perceptually distinct, but the decision to choose one or the other depends on one's inherent affinity for either one. In this case there is no correct answer, and it is equally correct to choose an apple or an orange. While these forms of decision-making may be distinct processes, real world problems often possess elements of both. The following behavioral experiment incorporates both of these elements of decision-making and looks at how they interact to produce behavior.

2.2. Results

2.2.1. Mice learn a 4-odor value-guided perceptual decision task

Olfactory tasks have proven to be an efficient way to study decision-making in rodents. This is because rodents can effectively discriminate many odors and learn contingencies that they predict in a variety of tasks. In this experiment I show that mice are capable of learning to discriminate amongst four odors that predict rewards of varying sizes (from now on referred to as reward values). Mice learn to self-initiate a trial and sample an odor, after which they select one of two options (fig. 2-1). All
animals in the experiment were capable of learning the correct contingencies for all four odors (fig 2-2). Despite the fact that the odors predicted rewards of different value, no differences were seen in the accuracy to each cue, or in the response time to any other the odor cues (fig 2-2, 2-5).

Given the role the amygdala plays in both encoding reward value and responding to changes in reward value, I was interested in understanding how this information about value might be incorporated to guide decisions. In the task described above, the correct actions associated with each odor are deterministic, in that each odor will only provide a reward for one of the two choices. In this sense, the task is a purely perceptual task, where the value of the expected reward does not provide any information about which choice will be correct. However, it is known that the expected value of a reward can bias an animal’s decision on a purely perceptual task.\textsuperscript{14, 15,} \textsuperscript{16, 17} In order to see this interaction of perceptual information and value information, I developed a categorization task similar to Wang et al., in which mixtures of the four odor types were delivered to the animal. The animals were only rewarded if they responded to the odor that was dominant in the mixture; the category. For example, if a mixture of 80\% odor A with 20\% odor B was given, then the animal would only be rewarded if it responded to the correct choice for odor A. The ratio of odors in the mixtures varied from pure odors, which were the easiest, to
60%/40% (from now on referred to as a 60/40 mixture), the most difficult. The main purpose for this was to create uncertainty in the stimulus presented, since mixtures that were closer to 50/50 category boundary were intrinsically more difficult. Since the odors being mixed could predict rewards of either equal or disparate value, this allowed me to look at the influence that value had on specific decisions. Animals are capable of learning the categorization task, and respond with varying accuracy to reward predicting cues. Although the value of a reward did not have any effect on the animal’s response times, there was a clear effect of difficulty in response times (fig. 2-5).

2.2.2. Expected reward value biases decisions in the direction of larger expected rewards

Animals were trained on the four odor binary mixture task after they had learned the contingencies for all pure odors. At this stage the animals had learned the correct action associated with each odor, as well as the reward value associated with each odor. The mixture task allowed me to look at the interaction between the perceptual information provided by the mixture, as well as the value information provided by each individual odor in the mixture.

Given that the task involved four odors of which four different mixture pairs could be created and mixed to varying degrees, I ended up with 24 different odor mixtures
which the animals had to respond to. In order to look at all of these parameters in a meaningful way, I developed a regression model that takes into account the difficulty of the mixture and the relative imbalance in reward value. Mixtures were categorized as either being balanced, in which both odors in the mixture predicted the same reward value (small/small, large/large), or imbalanced, in which one of the odors predicted a reward value larger than the other (small/large, large/small). These categories were also introduced into the regression model as categorical predictors. Taking into account the difficulty and reward value of each mixture I was able to show that indeed the expected reward value had an influence on the animal’s decisions. In cases where the expected reward was larger on one side, the animal was biased towards that side, despite the fact that this did not increase its chance of obtaining a reward. This effect also depended on the difficulty of the choice being made. On choices in which more perceptual uncertainty existed, the animal was more likely to be biased in the direction of the larger expected reward (fig 3-6).

2.2.3. Prior choices and outcomes bias decisions in the direction of correct prior choices

In trial and error learning, information is constantly being updated. With each new trial an animal must track what outcomes have occurred and what actions led to those outcomes. This mechanism requires a memory for past actions which must be
incorporated into current choices. Without such a mechanism, it would be difficult to adapt to changing contingencies in the world, and in fact many computational models of learning also require such a mechanism. To see if I could detect this mechanism being used in this specific task, I modified the regression model to incorporate a parameter representing the memory of past events. In this instance the parameter represents only the event that occurred in the previous trial (n-1). This takes into account the action that the animal took on the previous trial, as well as the outcome of that action. I then use this information to predict what the animal will choose on the following trial. Surprisingly, the information of this past trial has some predictive power on the subsequent trial, indicating that the animals are using this information to inform future choices. More specifically, the past trials that were rewarded bias the following trial in the direction in which that past reward was received. This can be differentiated from a win-stay lose-shift strategy in that the trials which were incorrect do not have any influence on subsequent trials (fig 2-7).

2.3. Discussion

In this task I attempt to mimic real world decision-making by incorporating elements of both perceptual and value-based decision-making. The animals had to learn to distinguish olfactory cues in order to determine which action would lead to a
reward—a perceptual choice with only one correct answer. However, since the cues also predicted the value of the reward—information which was not relevant to the perceptual choice—the animals also had the option to make a choice based on expected reward value. I showed that although value information was not relevant for determining the correct choice, it did exert an influence on current choice. This can be seen as an advantageous strategy in some sense because it allows other types of information to be utilized in cases where perceptual information is weak. One model suggests that a bias of fixed magnitude can actually maximize the amount of reward when choosing between unequal reward values. For example, if a hypothetical mixture of 50/50 were implemented which gave rewards randomly to either choice, it would be advantageous to always pick the choice of larger expected reward so that at least when a reward does arrive, it will be of larger value.

Another factor that I see exerting an influence on current decisions is the outcome of the most recent choice. By incorporating the outcome and decision of the previous choice into our regression model, I was able to detect and quantify the effect that this has on current decisions. This effect might seem surprising at first since the trials are independent from each other, and the previous trial has no predictive power for the subsequent trial, in the same way that flipping a coin once has no ability to predict the outcome of the next coin flip. However, this is consistent with reinforcement
learning models which update the probability of performing an action based on a weighted average of previous actions and their outcomes.\textsuperscript{20,21} This is not only necessary for learning to occur, but even after learning has occurred, this mechanism can be used to track any changes in the environment. A specific example of this is reversal learning, where the reward contingencies are reversed. In this scenario if a cue predicted a reward on the left and now predicts reward on the right, the animal must be able to track these changes. These can be tracked by incorporating a memory of the outcomes in which the left choice did not lead to a reward and in which right choices now are leading to reward. Without this type of outcome tracking it would difficult for the animal to behave flexibly when contingencies change. The amygdala is known to track the value of outcomes, and these signals are critical for proper encoding in downstream structures, such as the orbitofrontal cortex (OFC), which are critical for behavioral flexibility.\textsuperscript{6} Although there is confounding evidence regarding the role of the amygdala in cognitive flexibility, there is recent physiological evidence that the amygdala does encode changes in contingencies during a discrimination reversal task. Specifically, the amygdala encodes the trial to trial strategy, a win-stay lose-shift strategy, during learning.\textsuperscript{22}

The amygdala is a structure that is critical for many forms of learning, and more recent evidence suggests that it is specifically involved in tracking value across time.
The task developed in this chapter suggests that both value and outcome history play a crucial role in decision-making. The remainder of this thesis will investigate the role that the amygdala plays in these processes.

2.4. Methods

2.4.1 Animal Housing

Mice were singly housed in plastic home cages with laboratory bedding in a reverse 12:12 hour light/dark cycle room. All experiments were conducted in the dark phase of the light cycle. Experimental animals and littermate controls were started on behavioral training at 8-10 weeks of age.

2.4.2 Animal Water Regulation

Animals were placed on a water regulation schedule in order to motivate them to perform for water rewards. Water access was gradually reduced over the course of a week, after which 1.25 mL of water was provided daily. The animals were assessed for dehydration and body condition by the investigator and by veterinary technicians from the department of comparative medicine (DCM) at the Massachusetts Institute of Technology (MIT). Body condition measurements were performed in accordance to Ullman-Culleré et al., and animals were not allowed to fall below a body condition
score (BCS) of 2. Animals were assessed for dehydration by pinching the skin on the lower back and looking for a loss of elasticity in the skin. If an animal failed to meet these criteria, or if their body weight fell below 15% of their initial weight, then additional water was provided to ameliorate their condition.

2.4.3 Animal Training

**Shaping:** Animals were gradually shaped to perform an odor-guided two-alternative forced choice task. To begin, animals were trained to approach the reward ports of the operant chamber using a variable interval reward delivery schedule. Reward delivery intervals were chosen from a uniform distribution ranging from 3 seconds to 6 seconds. Animals were moved on to the next behavioral schedule when a minimum of 100 rewards were collected. Animals were then trained to initiate a trial by nose poking into the center port for a minimum period of time. The required poking time was gradually increased from zero seconds to 0.2 seconds, and finally to a uniformly distributed time between 0.2 and 0.3 seconds. A successfully initiated trial was indicated by all poke lights becoming lit; this signaled the availability of reward at either side port. At this point a successful poke to either side port delivered a water reward. After animals successfully collected 100 rewards in one session, they were moved on to the next phase of training. Using the same contingencies, air flow was
introduced into the center port, and gradually increased to a flow rate of 1000 mL/min. Once the animals were accustomed to a flow of 1000 mL/min and odorant (isoamly-acetate) was added at a flow rate of 60 mL/min.

**Two odor alternative forced choice:** After acclimation to the presence of an odor the reward contingencies were changed. Each successful trial initiation poke to the center delivered one of two possible odorants (hexanoic acid or hexanol), each of which predicted the presence of reward on one of the side pokes. The two odors were presented in a pseudo-randomized fashion across each trial. The animals were then kept on this protocol until they successfully learned the correct associations for the two odors. Successful learning was determined by a threshold of 70% correct in a completed session (100 trials).

**Four odor alternative forced choice:** After reaching the learning criteria for the two odor alternative forced choice task, an additional two odors (citral and cineol) were added to the possible odors presented to the animals. These odors also predicted a reward at either the left or right port, and additionally also predicted the presence of a larger reward. All four odors were then presented in a pseudo-randomized order. Learning was determined by a criterion of 70% correct on each of the four odor cues.
Four odor value-guided mixture task: After animals learned to correctly associate all four odors from the four odor alternative forced choice task to their correct choices, the odors were then mixed in pairs consisting of one odor indicating a left choice and one odor indicating a right choice (fig 3-1). The correct choice for each mixture was determined by the odor that composed the majority of the mixture. For example, a mixture of 80% Odor A with 20% Odor B indicates that the correct choice is the choice indicated by Odor A. All mixtures were equally likely and were pseudo-randomly presented.

2.4.4 Analysis of behavioral data

Behavioral data were analyzed using several regression models to fit the animal’s choice behavior. Since all choices were binary choices, a logistic regression model was used in all cases. The principal feature (regressor) used in all cases was the identity of the odor mixture. The size of this parameter will be used to assess the strength of the association between a given odor and its respective choice. Two additional dummy variables were used to represent the three-tiered categorical feature of the difference in size of the expected reward (left larger, right larger, or equal sized). A fourth categorical variable was used to represent the intersection of
the previous choice (n-1) with the previous outcome (1: n-1 correct on the left, -1: n-1 correct on the right, 0: n-1 incorrect) (equation 2-1).
2.5. Bibliography


19. Hueske, E. A role for Dopamine neuron NMDA receptors in learning and decision-making. (Massachusetts Institute of Technology, 2011).


2.6. Figures

Figure 2-1

a. 

b. 

<table>
<thead>
<tr>
<th>odor port</th>
<th>choice port</th>
<th>reward delivery</th>
<th>error feedback</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. 

Right Odor (%) 0 25 40 60 75 100

odor port choice port reward delivery error feedback
Figure 2-2

Accuracy Average

N = 7

% Correct

Mixture Difference (Left-Right)
Figure 2-3

Accuracy Small/Small

Accuracy Large/Large

Mixture Difference (Left-Right)
Figure 2-4

Accuracy Large/Small

Accuracy Small/Large
Figure 2-5

a.

Mixture Difference (Left-Right)

Response Times
Figure 2-6

a. N = 7 n = 20,392
- left larger
- same size
- right larger

b. Coefficients (β)

- Innate Bias Discrimination

- Left Bias
- Right Bias

% Left Choice
Concentration Difference (Left-Right)
Figure 2-7

- n-1 correct left
- n-1 correct right
- n-1 incorrect

P (Left Choices)

Concentration Difference (left - right)

Coefficients (β)

n-1 Choice-Outcome
2.7. Figure Legends

Figure 2-1: Decision-making task structure.
(a) Spatial configuration of the task: animals self-initiated a trial by poking into the center odor port, upon which an odorant was delivered. A successful poke based on the learned associations, a choice was then selected by poking into either the left or right water ports. A correct choice was resulted in a water reward. An incorrect choice resulted in the poke lights being turned off until the start of the next trial. (b) Temporal sequence of a trial: The poke into the odor port that satisfied the poke contingencies (see methods) started a trial, after which a poke to a choice port determined the animal’s choice. On correct choices, a water reward was delivered with a delay of 200-300 ms. On incorrect choices, immediate feedback was given in the form of the lights being turned off, and the implementation of a timeout period in which a trial could not be started. (c) Schematic of the odor mixtures used in the task. The odors were mixed in four possible combinations (small/small = light blue/light pink, large/large = dark blue dark red, large/small = dark blue/light pink, small/large = light blue/dark red)

Figure 2-2: Response accuracy across stimuli
Response accuracy is plotted for different odor mixtures. X-axis is the concentration
difference between odors for left and right responses. “1” indicates pure left odor and “-1” indicates pure right odor. The data correspond to the first 10 sessions of learning for the mixture task, and error bars represent the variance across animals (2xSD). Responses to pure odors are the most accurate at around 85% accuracy. As mixtures become closer to the category boundary (mixture difference = 0) the stimuli become more difficult to categorize and hence a drop in accuracy is seen.

Figure 2-3: Response accuracy by mixture type (balanced mixtures)
Response accuracy is separated by mixture types to make sure that animals can discriminate between all odors equally. (a) Response accuracy for mixtures with odors indicating small rewards on both left and right. (b) Response accuracy for odor mixtures indicating large rewards on both left and right. Both graphs indicate that the animals can discriminate the two sets of odors with approximately the same accuracy. There is a small but non-significant increase in the accuracy to odors indicating larger rewards.

Figure 2-4: Response accuracy by mixture type (unbalanced mixtures)
Response accuracy is separated by mixture type to make sure animals could discriminate all odors equally. On pure odors animals perform at an equal level of
accuracy, although a non-significant trend towards higher accuracy for the larger reward cue is seen. For mixtures, it is clear that differences exist that depend on the size of the reward, where animals are more accurate towards the side offering a larger reward. This is due to the animal choosing the larger reward size much more frequently for these stimuli.

**Figure 2-5: Response times separated by mixtures**

Boxplots for response times for the different mixtures in the task. The response time is defined as the time from when a trial is self-initiated to the time the animal makes a response. (a) A boxplot for including all reaction times for each mixture type. Horizontal red lines indicate the median for each group. Blue boxes include data from the 25th to 75th percentile. Red crosses indicate the outliers. (b) In order to perform an ANOVA, outliers that were more than 2 standard deviations away from the mean were removed. A significant change in the response time is seen for mixtures of varying difficulty. An ANOVA looking at the effect of mixture type on response time shows a significant effect of mixture type ($F_{(5,19706)} = 6.11$, $p=0.0000117$).

**Figure 2-6: Logistic regression for effect of reward value**
A regression to model choice behavior in a binary choice task. (a) points represent the choice frequencies for the left choice separated by mixture type (gray = equal reward on each side, blue = larger reward on the left, red = larger reward on the right). Lines represent the model’s predicted choice for each category.
Chapter 3

RsPo2+ Neurons Are Required for Integrating Prior Outcomes into Current Decisions.

In this chapter I characterize a transgenic mouse line expressing the enzyme CRE recombinase under the RsPo2 gene promoter. I show that CRE expression is confined to the anterior basal nucleus of the basolateral amygdala complex, and that expressing an activated caspase using a CRE-responsive virus can efficiently ablate these neurons. Using this ablation technique, I show that RsPo2+ are required for rapid learning of a perceptual decision-making task. Using a logistic regression model, I demonstrate that this learning deficit is due to an inability to integrate choice history into current decisions, and not an inability to discriminate between reward values. This suggests a mechanism by which decisions are influenced by both outcome history and expected outcomes, where both of these influences are mediated by different neuronal populations.
3.1. Introduction

The amygdala plays an important role in learning about emotionally-salient events. Its role in associative learning has been well-characterized using lesion studies in several animal species.\textsuperscript{1,2} Specifically, the amygdala encodes the value of potential outcomes (see chapter 2). One other feature that is encoded by the amygdala is the valence of the outcome, meaning that the amygdala also encodes whether an outcome is desirable (positive) or un-desirable (negative). It encodes this by using two populations of neurons, a population that increases its firing rate as the value of a positive outcome increases, and one that increases its firing rate as the value of a negative outcome decreases. These two populations have recently been genetically identified by Kim et al. using an activity-dependent screen, and have been shown to be mutually inhibiting.\textsuperscript{3} Since both the value and valence of an outcome are required to properly encode an outcome, this suggests that a balance of both these populations is required to properly transmit information to downstream structures.

In this experiment we sought to investigate how this balance may play a role in proper decision-making. We hypothesized that functionally ablating one of these populations would disrupt the balance between them, and that proper value encoding would be impaired. Given our behavioral results, showing that the value of an outcome can heavily bias an animal’s perceptual choice, we hypothesized that any
bias introduced by the value of an outcome would be affected by this manipulation.

3.2. Results

3.2.1. Rspos BAC transgenic animal recapitulates the endogenous mRNA expression pattern of Rspos gene

Mouse transgenics have been used successfully in the past to study brain circuitry with increasing specificity.\textsuperscript{4,5} Using genetic elements, we can target not only brain structures, but cells of different types within a circuit. Several strategies have been developed to create transgenic animals that express exogenous elements, such as RNAs and proteins, in specific patterns. A very popular method, which we use here, is to create a bacterial artificial chromosome (BAC), a large genetic element, which is then incorporated into the mouse genome.\textsuperscript{6} The BAC contains most of the surrounding genetic elements for the gene whose expression pattern one wishes to mimic. Inserting this large piece of DNA into the genome will recapitulate the original expression pattern. However, endogenous expression is not always achieved through this method for several reasons: (1) not all components controlling gene expression can be incorporated into the BAC (promoter elements, enhancers, etc.), (2) not all components required for proper gene expression are known for a specific gene, and (3) neighboring elements near the BAC insertion site, can influence the expression of the BAC gene.
To resolve these issues, many BAC insertions are generated and checked against the endogenous expression pattern of the gene. To do this we employed a viral strategy to target cells expressing CRE under Rspo2 promoter elements, and verified that this was consistent with the known endogenous expression pattern (fig 3-1). We injected an AAV virus that expresses palmitoylated green fluorescent protein (palmGFP) under the control of CRE recombination into the amygdala of transgenic animals. We used palmGFP, a GFP molecule that becomes covalently modified by the cell with the fatty acid palmitic acid in order to tether the molecule to the membrane. This modification occurs naturally in some proteins in order to tether themselves to the cell membrane. As a result of membrane tethering, visualization of the neuronal processes is greatly enhanced (figure 3-3).

RNA in situ hybridization performed by the Allen Institute Mouse Brain Atlas project shows that endogenous expression of the Rspo2 gene is confined the basal nucleus of the amygdala (fig 3-1c). By injecting a CRE-responsive virus expressing palmGFP into the Rspo2 transgenic animal, we can recapitulate the expression in the basal nucleus. We see that cell bodies are present at the most anterior portion of the basal nucleus, and that cell body density decreases towards the posterior of nucleus (fig3-2). We then looked for neuronal processes in known targets of the basal amygdala. The strongest processes were intra-amygdaloid processes projecting
locally within the nucleus. Strong processes were also seen to the dorsal and ventral striatum, both major targets of the basal amygdala. Finally, long range projections were also seen to the orbitofrontal and medial prefrontal cortex, structures with high interconnectivity with the basal amygdala. Overall, expression pattern of the Rsop2-CRE transgenic animal is consistent with it being a marker for basal amygdala neurons.

3.2.2 taCasp3 virus specifically ablates Rsop2+ neurons

Using the same viral technique that was used to express palmGFP in Rsop2+ neurons, we expressed a conditionally active Caspase-3 enzyme (taCasp3). This enzyme when activated, initiates a cell’s apoptosis signaling pathway, leading to programmed cell death, while sparing neighboring cells that do not contain taCasp. By using this technique, we were able to target a lesion in the amygdala that is specific to Rsop2+ neurons. An AAV-rh8 virus was packaged with a construct expressing a bi-cistronic transcript with taCasp3 and the activating enzyme TEVp. Upon expression of both proteins in CRE+ neurons, casp3 will become activated and begin apoptosis within the cell (fig 3-4).7

The efficiency of this technique is dependent on the expression levels of CRE protein in these cells, as this will determine the amount recombination that occurs.
and hence the amount of taCasp3 that will be expressed within the cell. We verified that apoptosis was occurring efficiently in this subpopulation by labelling the Rspo2+ neurons by crossing the Rspo2-CRE transgenic line with a reporter mouse that expresses GFP in CRE+ cells.8 In this mouse we then injected the AAV-taCasp3 virus into the basal amygdala unilaterally. We can see in the brain of this mouse that GFP is present in the basal amygdala of the un-injected side, but is nearly completely gone where AAV-taCasp3 was injected, confirming that GFP cells have undergone apoptosis (fig 3-5). We then use this strategy to ablate Rspo2+ neurons and test how they may be involved in decision-making behavior.

3.2.3. Rspo2 neurons are required for learning a 4-odor value-guided decision-making task

Two groups of animals were trained on the 4-odor value-guided decision-making task (see methods). One group received a bilateral amygdala injection of AAV-rh8-casp3 (experimental), and the other a bilateral amygdala injection of AAV-rh8-palmGFP (control). After recovering from surgery both groups began training at the same start point. After shaping the animals to the protocol, learning was measured for both groups at the 10-day learning period. Although, both groups performed poorly on odor mixtures that were difficult, the experimental group showed a significant decrement in performance to pure odors (fig 3-6). The animals with
amygdala lesions were eventually able to improve their accuracy with more training, but did so at a much slower rate than control animals. This suggests that Rsbo2+ neurons are required for efficient learning of odor associations.

3.2.4. Rsbo2 neurons are required for the integration of choice history

Successful learning requires the proper integration of choice history in order to update associations (see chapter 2). To specifically identify the learning deficit that was seen when ablating Rsbo2+ neurons, we made use of the logistic regression model that was implemented in chapter 2. By using the logistic regression model we were able to factor out the influence of different features on the animal’s choice behavior. Given the amygdala’s known role in value encoding, we were particularly interested in how the value-induced bias might be affected by the lesion of Rsbo2+ neurons.

We performed a logistic regression which included as features: (1) the concentration difference in odors (β1), (2) two indicator variables for the categorical feature of relative reward size (B2, B3), and (3) a categorical feature for the choice and outcome of the previous trial (B4). The fit of feature coefficient β1 for both groups, confirms that the experimental group has not acquired the odor associations as much as the control group. The experimental group has a learning parameter (β1) that is
significantly lower than than the control group (95% CI = [1.6403, 1.7439] and [1.3505, 1.4401] respectively). This indicates that the model of choice behavior in the experimental group, is less able to predict the animal’s choice based on the odor mixture. This makes sense since the animal has not properly learned the correct association for each odor mixture.

Surprisingly, when we looked at the coefficient for the value-induced bias in both groups, we did not see any significant difference between groups ($\beta_2_{control}$ 95% CI = [0.6773,], $\beta_3_{control}$ 95% CI = [-0.6585, -0.4949], $\beta_2_{experimental}$ 95% CI = [0.80071, 0.9583], $\beta_3_{experimental}$ 95% CI = [-0.7633, -0.6181]). This suggests that $Rspo2^+$ neurons are not required for learning the value of rewards. However, when we looked at the coefficient for the feature encoding the prior choice and outcome, we see a strong difference in the size of this coefficient. In control groups, as we have shown in chapter 2, animals are biased by their prior choices and outcomes, which we capture here with the $\beta_4$ coefficient. In the control group the coefficient for this parameter has a coefficient within a 95% CI of [0.2036, 0.2852], whereas the experimental group has a coefficient value with a 95% CI of [0.0275, 0.1011], meaning that they have non-overlapping confidence intervals. This suggests that $Rspo2^+$ neurons are required for integrating information from prior trials into current decisions.
3.3. Discussion

Decision making requires the complex integration of multiple inputs into a system that produces behavior. As we can see from the regression model, there are several factors that can influence decisions, such as the value of an expected reward, and history of past choices. These influences manifest themselves as biases in what should be a deterministic choice, and can be measured by taking into account all of the factors that make up decisions. Much like the factors that influence decisions, the neural structures that are involved in decision making are also vast, and their unique role in the decision making process is relatively unknown. Several structures, such as the prefrontal cortex and nucleus accumbens, also encode the outcomes and the value of those outcomes, and how these interact with structures like the amygdala is still being uncovered.\textsuperscript{9,10} By specifically manipulating these circuits we can uncover their specific role in behavior.

When we specifically ablate a sub-population of cells in the amygdala we can very plainly see how this affects behavior when we look at the how well the animal is able to learn. The requirement of $Rspo2^+$ neurons in learning is not unexpected, since we have long known that the amygdala is required for associative learning. However, what is surprising is that although the animal is deficient in learning, the bias that is induced by the value of an outcome persists. This suggests that $Rspo2^+$ neurons are
required for learning the associations between the odor cues and their correct choice, but are not required for learning the association between these cues and their associated values, suggesting that these two functions are controlled by different circuits.

Rspos+ neurons have recently been implicated in aversive learning. This could explain why these neurons are not required for a value-induced bias since the encoding of reward value is being performed by an opposing population. However, the encoding of valence in this structure requires a balance of both positive and negative-encoding neurons, something that is abolished when Rspos+ neurons are ablated. Valence is a fundamental component to efficient learning. This predicts that ablating either the positive or negative-encoding population will create a learning deficit.

We looked more closely at the causes of this learning deficit by comparing different features of our regression model. When we look at how choice history influences behavior, we see that normally an animal's recent choice heavily influences their current choice. One critical component of efficient learning is the integration of prior outcomes and choices. This solves what is known as the temporal credit assignment problem, how an organism can detect which actions in the past led to a positive outcome and which outcomes led to a negative outcome. When we look at
this component after ablating Rspo2+ neurons, we see that this is completely
abolished, suggesting that one critical function of these neurons is to incorporate
recent choices into the present.

3.4. Methods

3.4.1 Animal Housing

Mice were singly housed in plastic home cages with laboratory bedding in a
reverse 12:12 hour light/dark cycle room. All experiments were conducted in the
dark phase of the light cycle. Experimental animals and littermate controls were
started on behavioral training at 8-10 weeks of age.

3.4.2 Animal Water Regulation

Animals were placed on a water regulation schedule in order to motivate them to
perform for water rewards. Water access was gradually reduced over the course of a
week, after which 1.25 mL of water was provided daily. The animals were assessed
for dehydration and body condition by the investigator and by veterinary technicians
from the department of comparative medicine (DCM) at the Massachusetts Institute
of Technology (MIT). Body condition measurements were performed in accordance
to Ullman-Culleré et al., and animals were not allowed to fall below a body condition
score (BCS) of 2. Animals were assessed for dehydration by pinching the skin on the lower back and looking for a loss of elasticity in the skin. If an animal failed to meet these criteria, or if their body weight fell below 15% of their initial weight, then additional water was provided to ameliorate their condition.

3.4.3 Surgery

**Pre/Post-Operative Care:** All surgeries were performed in an aseptic surgical facility. Anesthesia was induced with either an intraperitoneal injection of Avertin (Avertin Tribromoethanol at 20 mg/ml), or through a constant gaseous stream of isoflurane. The anti-inflammatory drug Metacam (Meloxicam) was given prior to surgery, and subsequently given daily for three days of post-operative care.

**Viral Infusion:** Hair on the scalp was removed using an electronic hair clipper. The scalp was disinfected using a Betadine solution before incision. An incision was then made along the anterior/posterior axis of the scalp, and the skin was pulled back to access the cranium. Using a mouse stereotaxic device from Kopf Instruments, the anterior/posterior and medial/lateral coordinates were marked on the skull. A craniotomy was made at these locations using a dental drill.

A pulled glass pipette attached to a Hamilton syringe, was used to deliver the virus into the brain. The syringe was attached to the stereotaxic device and lowered to the correct coordinate. A precision pump was then used to deliver the virus to the
injection site.

Caspase Virus Infusion: Spread of the virus maximized by infusing the virus at two different sites for each hemisphere.

<table>
<thead>
<tr>
<th>Coordinate</th>
<th>Infusion Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP -1.46, ML +3.3, DV -4.68</td>
<td>250 nl</td>
</tr>
<tr>
<td>AP -1.96 ML +3.3 DV -4.68</td>
<td>250 nl</td>
</tr>
<tr>
<td>AP -1.46 ML -3.3 DV -4.68</td>
<td>250 nl</td>
</tr>
<tr>
<td>AP -1.96 ML -3.3 DV -4.68</td>
<td>250 nl</td>
</tr>
</tbody>
</table>

3.4.4 Animal Training

Shaping: Animals were gradually shaped to perform an odor-guided two-alternative forced choice task. To begin, animals were trained to approach the reward ports of the operant chamber using a variable interval reward delivery schedule. Reward delivery intervals were chosen from a uniform distribution ranging from 3 seconds to 6 seconds. Animals were moved on to the next behavioral schedule when a minimum of 100 rewards were collected. Animals were then trained to initiate a trial by nose poking into the center port for a minimum period of time. The required poking time was gradually increased from zero seconds to 0.2 seconds, and finally to a uniformly distributed time between 0.2 and 0.3 seconds. A successfully initiated trial was
indicated by all poke lights becoming lit; this signaled the availability of reward at either side port. At this point a successful poke to either side port delivered a water reward. After animals successfully collected 100 rewards in one session, they were moved on to the next phase of training. Using the same contingencies, air flow was introduced into the center port, and gradually increased to a flow rate of 1000 mL/min. Once the animals were accustomed to a flow of 1000 mL/min and odorant (isoamly-acetate) was added at a flow rate of 60 mL/min.

**Two odor alternative forced choice:** After acclimation to the presence of an odor the reward contingencies were changed. Each successful trial initiation poke to the center delivered one of two possible odorants (hexanoic acid or hexanol), each of which predicted the presence of reward on one of the side pokes. The two odors were presented in a pseudo-randomized fashion across each trial. The animals were then kept on this protocol until they successfully learned the correct associations for the two odors. Successful learning was determined by a threshold of 70% correct in a completed session (100 trials).

**Four odor alternative forced choice:** After reaching the learning criteria for the two odor alternative forced choice task, an additional two odors (citral and cineol) were
added to the possible odors presented to the animals. These odors also predicted a reward at either the left or right port, and additionally also predicted the presence of a larger reward. All four odors were then presented in a pseudo-randomized order. Learning was determined by a criterion of 70% correct on each of the four odor cues.

**Four odor value-guided mixture task:** After animals learned to correctly associate all four odors from the four odor alternative forced choice task to their correct choices, the odors were then mixed in pairs consisting of one odor indicating a left choice and one odor indicating a right choice (fig 3-1). The correct choice for each mixture was determined by the odor that composed the majority of the mixture. For example, a mixture of 80% Odor A with 20% Odor B indicates that the correct choice is the choice indicated by Odor A. All mixtures were equally likely and were pseudo-randomly presented.

### 3.4.5 Analysis of behavioral data

Behavioral data were analyzed using several regression models to fit the animal’s choice behavior. Since all choices were binary choices, a logistic regression model was used in all cases. The principal feature (regressor) used in all cases was the identity of the odor mixture. The size of this parameter will be used to assess the strength of the association between a given odor and its respective choice. Two
additional dummy variables were used to represent the three-tiered categorical feature of the difference in size of the expected reward (left larger, right larger, or equal sized). A fourth categorical variable was used to represented the intersection of the previous choice (n-1) with the previous outcome (1: n-1 correct on the left, -1: n-1 correct on the right, 0: n-1 incorrect) (equation 3-1).

**Analysis of Caspase lesion experiment:** The first 10 sessions of the 4-Odor Mixture task were taken for each mouse in both groups for analysis. Each animal was trained on the 4-Odor Alternative Forced Choice task beforehand and was moved on when they reached the same criterion of 70% correct.
3.5. Bibliography


3.6. Figures

Figure 3-1

a. AAV-rh8

b. Rsop2 transgenic
Figure 3-2

Anterior

Posterior

a. 

b. 

c. 

d. 

e. 

f. 

g. 

h. 

i. 

j. 

AAV-DIO-palmGPP  NeuN  Merge
Figure 3-3
Figure 3-4

a.

AAV-rh8

+CRE

TEVp

Pro-taCasp3

taCasp3
Figure 3-5

AAV-DIO-taCasp3
Rspo2-CRE::rosa-Isl1-GFP
Figure 3-6

a. Control
   Rspon lesion
Figure 3-7

(a) = left larger
= same size
= right larger

N=9

Control

Concentration Difference (left - right)

(b) = left larger
= same size
= right larger

N=10

Caspase

Concentration Difference (left - right)
c. 

- = GFP control
- = taCasp3 lesion

![Coeficients Size vs Discrimination](image)

![Left Value Bias vs Right Value Bias](image)

n.s.: not significant
Figure 3-8

a.

- n-1 correct left
- n-1 correct right
- n-1 incorrect

GFP n-1 effect

\[ \text{Concentration Difference (left - right)} \]

b.

Caspase n-1 effect

\[ \text{Concentration Difference (left - right)} \]
C.

![Graph showing coefficients size vs. n-1 Outcome Bias with a star indicating a significant result.]

\[ n-1 \text{ Outcome Bias} \]

\[ \beta_4 \]
3.7. Figure Legends

Figure 3-1: Neuronal labelling strategy

(a) AAV virus used for labelling CRE-expressing neurons. Palmitolated GFP was used to tether GFP to the membrane and label cell bodies and projections. Expression of GFP was constrained to CRE-expressing cells by using a Double-floxed Inverse Open reading frame (DIO) and a human synapsin promoter. (b) Virus was stereotaxically injected into the amygdala where CRE expression was localized in the Rspo2 transgenic line. (c) mRNA expression pattern of Rspo2 gene in mouse brain from Allen Brain Atlas

Figure 3-2: Rspo2-CRE localization within the amygdala

Anterior to posterior distribution of CRE expression within the amygdala using DIO-palmGFP virus and co-labelled with neuronal marker NeuN. (a,b,c) Anterior portion of the amygdala shows expression throughout the basal nucleus of the amygdala. GFP expression within cell bodies is confined to the basal nucleus, whereas projections are seen locally. (d,e,f) Intermediate section of the amygdala shows that CRE is confined to the basal nucleus and projections appear to circumvent the central nucleus of the amygdala. (g,h,j) Much less expression is seen in posterior sections of the amygdala.
Figure 3-3: Projections of Rspo2-CRE neurons

(a) Coronal section of GFP expression within the amygdala. Projections to the medio-dorsal thalamus (MD Thalamus) and dorsal striatum are seen. (b) Sagittal section showing projections near the amygdala and to the dorsal striatum. (c) Horizontal section showing expression within the basal nucleus and projections to the ventral striatum, and ventral hippocampus. (d) Anterior coronal section showing projections to the prefrontal cortex.

Figure 3-4: Rspo2+ lesion strategy

(a) Virus construct using the human synapsin DIO cassette. CRE recombinase expression will activate the cassette by inverting the ORF. The ORF contains two ORFs expressing modified pro Caspase3 enzyme and TEVp enzyme. TEVp is an enzyme that cleaves proCaspase3 and activates it. Activated caspase will initiate the neuron’s inherent apoptosis pathway and leads to self-destruction.

Figure 3-5: Rspo2+ caspase lesion

(a) Expression of GFP using a double transgenic approach. A double transgenic line was created using Rspo2-cre transgenic line and the CRE-responsive GFP line (ai9).
Left amygdala (white outline) was injected with AAV-rh8-DIO-taCasp3. GFP is expressed in cells containing CRE. On the left side where taCasp3 is also expressed, cells that were GFP+ have been ablated.

Figure 3-6: Response accuracy of Rspo2-lesioned animals
(a) Accuracy on first 10 sessions for control and Rspo2-lesioned animals. Control animals show a maximal accuracy of ~90% on pure odors (mixture difference = -1,1) which decreases to around chance for the most difficult mixture. Lesion animals follow the same pattern, but obtain a much lower accuracy for all mixtures. A two-way ANOVA for effects of difficulty ($F(5,353) = 18.29, p=3.75e^{-16}$) and lesion ($F(1,353) = 41.33, p=4.18e^{-10}$) shows that both significantly differentiate the data.

Figure 3-7: Logistic regression for effect of reward value
(a,b) Choice data and regression for the control and caspase-lesion group (injected with GFP virus). Points represent the percentage of choices to the left choice for a given concentration difference and reward value. Percentage of left choices increases with increasing left-predicting odors, indicating that the animals have learned the mixture task. In addition, a shift towards the left and right (bias) are seen when the respective side predicts a larger reward. Left and right biases are seen in the caspase-
lesion group as well, although the slope of the regression curves is smaller due to the fact that the group has not learned the associations as well. (c) Plot of the discrimination parameter ($\beta_1$) for both groups shows a significant decrease in the parameter size for the lesion group. (d) Plots of the parameters for the leftward and rightward value bias ($\beta_2$, $\beta_3$) show no difference between control and lesion group, indicating that both are equally biased by relative reward size.

Figure 3-8: Logistic regression for effect of prior outcome

(a) Logistic regression curves for the GFP control group showing the probability of left choices for each mixture type. Each curve is the predicted probability of choosing left color-coded for the previous outcome (n-1). Animals are biased to the left or right when the previous choices were rewarded at the left or right respectively. (b) The same logistic regression on the caspase-lesion group shows that the bias produced from previous choices is not present. (c) A plot of the coefficient sizes for the feature of n-1 outcomes. A significant difference is seen in the size of these coefficients, where the lesion group shows a much lower influence of the previous outcomes.
Chapter 4

Putative Rspo2+ Neurons Encode Prior Outcomes Among Other Decision Variables.

In this chapter I perform live imaging of regions of interest (ROIs) in Rspo2+ neurons in behaving animals using a virally-delivered GCaMP protein in combination with a head-mounted miniature endoscope. By imaging Rspo2+ ROIs in vivo, I was able to capture the dynamic encoding of these ROIs while the animal learns a value-guided decision-making task. I show that Rspo2+ ROIs are inhibited by reward-predictive cues, and are activated in the inter-trial interval (post-reward period), consistent with its recently described role of Rspo2+ neurons in fear learning. In addition, the activity in the inter-trial interval is modulated by prior outcomes, suggesting a mechanism by which these putative neurons may be used to integrate information across trials. To distinguish the activity of Rspo2+ ROIs from other neuronal activity, I also imaged ROIs using a pan-neuronal promoter to deliver the GCaMP protein into multiple neuron types.
4.1 Introduction

Distinct subsets of neurons have been shown to exist within the amygdala.\textsuperscript{1,2} These have been characterized by using traditional electrophysiology techniques, which have described two main populations that respond to aversive and rewarding outcomes respectively. More recently, studies have confirmed that these two populations are genetically distinct and mutually-inhibiting, suggesting a mechanism by which the balance of activity is used to produce behavior.\textsuperscript{3-5} One subset of these neurons, expressing the gene Rspo2 is specifically involved in fear learning. As discussed earlier, these neurons are also required for proper decision-making, specifically for the integration of prior outcomes during learning (chapter 3).

To further investigate the mechanism by which these neurons participate in the decision-making process, we performed live imaging during learning. By combining several technologies, we were able to specifically perform live imaging of putative Rspo2+ neurons while an animal learned a decision-making task. We utilized the CRE-LoxP system that we have used to manipulate these neurons and combined it with the GCaMP fluorescent imaging system. The GCaMP protein is a modified version of the fluorescent protein GFP, which has been constructed to fluoresce only under conditions of elevated calcium ions, a critical ion in neuronal signaling. The mechanism of signal transduction between neurons, the action potential, necessitates
an influx of calcium ions into the neuron, which can be detected by the presence of GCaMP. This allows us to use the fluorescence of GCaMP as a proxy for neuronal cell activity.\textsuperscript{6} Capturing fluorescence from these ROIs requires the use of a microscope, which poses a problem when an animal must move around to perform a task. To overcome this, we made use of a miniaturized microscope developed by Inscopix.\textsuperscript{7} This is a miniature imaging device that can be attached to the animal and is small enough for the animal to carry while performing the task.

We were particularly interested in the dynamics of neuronal activity in Rspl2+ neurons as the animal learned the task, as we have shown that these neurons are required for learning of a decision-making task. Studies have shown how subsets of amygdala neurons encode reward-predictive cues, and how this activity develops through learning, but these have been in relatively simple tasks in which cue associations are acquired very quickly, and in which no uncertainty is present in those cue associations.\textsuperscript{8,9} By using a decision-making task in which there is uncertainty present, we assure that learning must take place over a longer period of time, allowing us to capture the dynamics of learning. Additionally, by labelling specifically Rspl2+ neurons we can further characterize the role of these putative neurons in learning.
4.2 Results

4.2.1. A subset of Rspre2+ ROIs is inhibited by reward-predicting cues

We combined the CRE-LoxP system with GCaMP imaging in awake behaving animals in order to capture the dynamics of neuronal activity as learning occurs (fig 4-1). By combining these systems, we were also able to distinguish the activity of genetically distinct populations, and show that Rspre2+ ROIs have an activity pattern distinct from other amygdala ROIs. Using this method, we were able to observe many ROIs simultaneously and detect their activity (fig 4-2). One activity pattern that we observe is the inhibition of a subset of ROIs to the presentation of a reward-predicting odor cue. By aligning the events of all ROIs recorded to the onset of the onset of odor sampling, we were able to observe a significant decrease in the event rate (fig 4-3). This is a pattern that is observed in neurons that encode aversive outcomes, and is expected given the role of Rspre2+ neurons in fear learning.

In order to look at the effects of learning we wanted to compare neuronal activity within the same ROI to test whether this activity was changing over time. Specifically, we were interested in how this activity was modulated on a trial-by-trial basis. To do this we subset the specific activity of a ROI into one subset in which the previous trial was rewarded and one in which the previous trial was an error. Trials which were preceded by an aborted trial were removed from the analysis. For example, for ROIs
that were inhibited by cues, we took the activity within a 4-second window around the cue, and subset that activity into the aforementioned groups. We then performed and **ANOVA** on the average rate of events between these two groups. For these neurons, there was no significant difference between groups, suggesting that these neurons are not modulated by the outcome of the previous trial (fig 4-3f).

In addition to recording from *Rspo2*+ ROIs we were interested in ROIs that did not express *Rspo2*. We were able to image these additional ROIs by instead using a virus that expressed GCaMP constitutively under the control of the human synapsin promoter (hsyn), and therefore expresses in all infected neurons. This virus expresses GCaMP in all neuronal cell types, allowing us to capture additional ROIs (fig 4-5). One subset of ROIs that we can observe in this group of animals that is not present in the *Rspo2*+ subset, are ROIs which respond to reward-predictive cues by increasing their firing rate (fig 4-6). These neurons have a typical response of neurons that are involved in reward learning. Due to a low number of these ROIs in our recordings however, we did not perform any further group analysis on this subset of ROIs.

### 4.2.2. A subset of *Rspo2*+ ROIs are activated after reward delivery and presumably reward consumption

A second activity pattern that we observe in *Rspo2*+ ROIs, is an increase in activity in the inter-trial period. The increase occurs after the animal has made a correct
choice and presumably consumed the water reward, on average 0.25 seconds after the delivery of a reward (fig 4-4). This is in contrast to a subset of ROIs when recording all amygdala neurons, where activity increases sharply at the point of correct port entry (fig 4-7). We were not able to measure the exact time point at which the reward was consumed, but we assume that given the miniscule size of the reward, that 0.25 seconds would be enough time to consume it. We aligned the activity to the exit time from the choice port, but activity appears to precede this time point (not shown). This is not surprising as it is common for mice to continue to poke into the reward port even after the reward has been consumed.

The precise timing of these ROIs in the period between reward consumption and the subsequent trial suggests that these putative neurons may be responsible for communicating outcome information from one trial to the next. To further investigate this, we subset the activity based on the previous outcome. What we see is that on average the activity of these ROIs in the inter-trial period appears to be higher during periods in which the previous trial was a correct choice. Using an ANOVA test we see that the average activity is different between these two groups (fig 4-4c). This subset of neurons is not only active during the inter-trial period, but is also modulated by previous outcomes, implying a mechanism by which outcome information is transferred from trial to trial during learning.
4.2.4 Amygdala ROIs are responsive to correct choices

We wanted to test whether this modulation by previous outcomes occurred in other populations. We could not detect any ROIs that showed an increase in activity to correct choices in the population labelled by Rspo2, but were able to detect these in the group of amygdala ROIs labelled using a pan-neuronal promoter. These ROIs show a sharp increase in event rate upon entry to a correct choice port. When we subset this activity based on the previous trial, we also see a modulation by trial history (fig 4-8). We see that on average the activity is significantly higher for trials which were preceded by correct choices, however this is at a different time point than the ROIs described in the Rspo2+ case. This suggests that modulation by trial history might be a general mechanism used within the amygdala to update cue associations that may be constantly changing.

4.3 Discussion

By using the GCaMP imaging system along with the Inscopix miniature microscope, allowed us to confirm some of the activity that has previously been reported in amygdala recordings. Because of the mechanism by which this method works, this is only a proxy for neuronal activity, which has an inherently lower
sampling rate, giving us much lower temporal resolution. However, by recording from a general population of amygdala ROIs, we were able to confirm that a subset of ROIs increases activity to both the reward-predicting cue, and to correct choices leading to rewards.

Another caveat with this method of imaging is that since it involves a one photon microscope, the focal plane of the image is not restricted to a single neuronal plane. This means that neuronal activity is being captured from multiple focal planes, leading to cross talk between different sources of signal. This can occur in the form of neurons that are overlapping, neuronal processes that are passing through, neuropil activity, or even background noise. The method we used to sort putative neurons was manual selection of ROIs based on visual inspection of the images. ROIs were selected based on morphology that resembled a neuron, and a criteria of non-overlapping ROIs. While this method allows us to distinguish neurons that are partially overlapping, it does not permit us to detect differences in activity from highly overlapping cells, and neuropil activity that might coincide with the ROI. Other methods have been employed to ameliorate this problem, such as principal component analysis (PCA). PCA is a form of dimensionality reduction that works by finding correlations in the dimensions of the data, in this case the pixels of the image across time, and selecting the dimensions that preserve the highest variance. Since
background noise is perfectly uncorrelated, this can often be removed by discarding
the first principle components. However, since activity in the amygdala if often
coordinated, leading to low variability in the pixels between ROIs, these will often be
captured by the same principle component. In this sense, PCA is not a good method
for sorting individual cells, since a single component will often contain the pixels of
multiple cells if they are coordinated. Independent component analysis (ICA) is
another form of dimensionality reduction, which has been used with success in
identifying different sources of signal. However, this method assumes
independence between signals, which is not the case when neural activity is
coordinated. For this reason, we use a manual ROI selection method, with the caveat
that multiple signal sources might be present in any specific ROI.

By specifically imaging RsPo2+ ROIs we were able to detect an inhibition of these
ROIs as the animal sampled a reward-predicting cue, bolstering the claim that these
putative neurons are specific for encoding the aversive features during learning. The
inhibition of these ROIs to reward predictive cues is suggested by the fact that
RsPo2+ neurons have been shown to be inhibited by another subset of neurons that
is primarily involved in reward learning.

One other activity pattern that we uncovered in a subset of RsPo2+ ROIs showed
an increased activity during the inter-trial period. This activity peaked at 0.25-0.75
seconds after reward. One reason this might occur is due to the aversive effect of the absence of reward once it has been consumed. More interestingly, this activity occurs between subsequent trials during the task, suggesting it might be important for integrating information between trials as the animal learns. We looked for evidence of this by sub-setting this activity based on the outcome of previous trials, and showed that looking at the average activity of these ROIs, they behave substantially different depending on what outcome occurred most recently. These ROIs were more active in trials where the previous choice led to a correct action, suggesting a type of memory for the correct action in the previous trial. This is a critical mechanism for trial and error learning.

We were also able to detect a similar modulation of activity in a subset of ROIs in which the activity at the time of a correct choice is modulated by the previous trial outcome. These ROIs are involved in the encoding of rewarding outcomes, and the fact that modulation of activity between trials occurs in both types of cells suggest a general mechanism by which amygdala neurons update behavior as learning occurs. This type of modulation is similar to the activity seen in other brain structures.12,13

4.4 Methods

4.4.1 Surgery
Pre/Post-Operative Care: All surgeries were performed in an aseptic surgical facility. Anesthesia was induced with either an intraperitoneal injection of Avertin (Avertin Tribromoethanol at 20 mg/ml), or through a constant gaseous stream of isoflurane. The anti-inflammatory drug Metacam (Meloxicam) was given prior to surgery, and subsequently given daily for three days of post-operative care.

Viral Infusion: Hair on the scalp was removed using an electronic hair clipper. The scalp was disinfected using a Betadine solution before incision. An incision was then made along the anterior/posterior axis of the scalp, and the skin was pulled back to access the cranium. Using a mouse stereotaxic device from Kopf Instruments, the anterior/posterior and medial/lateral coordinates were marked on the skull. A craniotomy was made at these locations using a dental drill.

A pulled glass pipette attached to a Hamilton syringe, was used to deliver the virus into the brain. The syringe was attached to the stereotaxic device and lowered to the correct coordinate. A precision pump was then used to deliver the virus to the injection site.

**AAV-DIO-GCaMP6f Virus Infusion:** The spread of the virus was maximized by infusing the virus at two different sites on one hemisphere. Coordinates: (1) AP -1.46, ML+3.3, DV -4.68, (2) AP -1.96 ML + 3.3 DV-4.68.

**AAV-hsyn-GCaMP6f Virus Infusion:** Viral spreading in this situation had to be
minimized due to the fact that expression was not limited to CRE+ cells. We injected a 200 nl of virus into one site AP -1.46, ML+3.3, DV -4.68.

**Miniature microscope surgery:** Imaging was performed using an Inscopix miniature microscope system along with NVista software. After a 4-week incubation period, the mice that were injected with GCaMP virus underwent a second surgery to implant a lens probe into the amygdala. The lens probe was lowered into the same craniotomy from the viral injection and permanently fixed onto the head of the mouse using miniature metal screws and dental cement. After a second 4-week incubation period, the lens was tested for activity by placing the miniature microscope above the lens probe. Animals that showed any fluorescence were fixed with a baseplate to attach the microscope in future sessions. After selecting animals with fluorescence, this group of animals began training on the decision-making task.

4.4.2 Animal Training

**Shaping:** Animals were gradually shaped to perform an odor-guided two-alternative forced choice task. To begin, animals were trained to approach the reward ports of the operant chamber using a variable interval reward delivery schedule. Reward delivery intervals were chosen from a uniform distribution ranging from 3 seconds to 6 seconds. Animals were moved on to the next behavioral schedule when a minimum
of 100 rewards were collected. Animals were then trained to initiate a trial by nose poking into the center port for a minimum period of time. The required poking time was gradually increased from zero seconds to 0.2 seconds, and finally to a uniformly distributed time between 0.2 and 0.3 seconds. A successfully initiated trial was indicated by all poke lights becoming lit; this signaled the availability of reward at either side port. At this point a successful poke to either side port delivered a water reward. After animals successfully collected 100 rewards in one session, they were moved on to the next phase of training. Using the same contingencies, air flow was introduced into the center port, and gradually increased to a flow rate of 1000 mL/min. Once the animals were accustomed to a flow of 1000 mL/min and odorant (isoamly-acetate) was added at a flow rate of 60 mL/min.

Two odor alternative forced choice: After acclimation to the presence of an odor the reward contingencies were changed. Each successful trial initiation poke to the center delivered one of two possible odorants (hexanoic acid or hexanol), each of which predicted the presence of reward on one of the side pokes. The two odors were presented in a pseudo-randomized fashion across each trial. The animals were then kept on this protocol until they successfully learned the correct associations for the two odors. Successful learning was determined by a threshold of 70% correct in a
completed session (100 trials).

Two odor mixture task: After reaching criterion for the two odor alternative forced choice task the animals were moved on to an alternative forced choice task involving mixtures of the two odors. Imaging data was collected during this phase.

4.4.3 Imaging Data Analysis

The imaging data was collected with the NVista hardware from Inscopix Inc., and was initially processed using the software package Mosaic. Mosaic software was used to correct for motion that occurs as the animal moves.

Subsequent image processing was performed using ImageJ. Image stacks were first filtered using a bandpass filter to reduce spatial noise in the image. The image stack was then normalized by dividing by the average luminance in the stack at each pixel position. The final image stack contained pixels representing the change in luminance relative to the average ($\Delta F$). $\Delta F$ values were then exported into a spreadsheet to be analyzed using MATLAB. Regions of interest (ROI) were selected manually where neuronal activity was detected. Regions were selected to be of equal size (4 pixels); this minimized the potential overlap of the ROIs and the noise associated with any motion of the ROI. The regions were selected based on their
shape (circular) and if they contained neuronal processes. They also had to contain at least one event, defined as a deflection in ΔF of at least 2 SD.

4.4.5 ROI Selection

All ROIs that were selected for further analysis were tested for event-related activity. To do this we used a heuristic measure around the time of the event being aligned. A 0.5 seconds window before and after the event was used to test for cue and reward related activity. All neurons included in further analysis had at least a 50% change in event number between these two windows. For activity classified as inter-trial activity, the selected neurons were tested with a 0.5 seconds window before the reward and window between 0.5-1 second after the reward and the same test of 50% change was applied

4.5 Bibliography


Figure 4-1

a. 
b. 

odor port
choice port
reward delivery
error feedback
Figure 4-2

a. b.
Figure 4-3

a. 

Rs-po2+ Example Neuron
Cue Inhibition

- Cue
b. 

*Rspo2+ Example Neuron*

Event Rate (hz) vs. time (s)
RsPo2+ Example Neuron

Cue →

Event Rate (Hz)

-2 -1.5 -1 -0.5 0 0.5 1 1.5 2

Time (s)
RsPo2+ Example Neuron

Event Rate (Hz)

time (s)
$Rspo2^+$ Average Response

N=4

Event Rate (Hz)

time (s)

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0

-1.5 -1 -0.5 0 0.5 1 1.5
RsPo2+ Average Response

ANOVA
p > 0.9
N = 4

n-1 correct / n-1 incorrect

Average Rate (Hz)

Cue

n-1 correct
n-1 error

Average Rate (Hz)

-2 -1.5 -1 -0.5 0 0.5 1 1.5 2 2.5 3 3.5

f.
Figure 4-4

a.
b. RsPo2+ Inter-Trial Response

Reward →

Event Rate (Hz)

-2 -1.5 -1 -0.5 0 0.5 1 1.5 2

time (s)
Rspo2+ inter-trial response average

n-1 correct / n-1 incorrect

ANOVA
F = 6.57, p=0.0121
F = 8.18, p=0.0053
N= 8

reward →

Average Rate (Hz)

-2 -1.5 -1 -0.5 0 0.5 1 1.5 2

N-1 correct
N-1 error
Figure 4-5

a. AAV-rh8

b.
Figure 4-6

a. Whole amygdala cue response
Whole amygdala cue response (example)
Figure 4-7

a. Whole amygdala correct choice response (example)
Whole amygdala correct choice response (example)
Whole amygdala correct choice response (average)

Event Rate (Hz)

correct choice

-1.5 -1 -0.5 0 0.5 1 1.5

time (s)
Figure 4-8

a. Correct choice (n-1 correct)

b. Correct choice (n-1 error)
Amygdala Correct Choice Response Average

ANOVA

F = 13.33, p = 0.0004

N = 10

Correct Choice

n-1 Correct

n-1 Error

Average Rate (Hz)

-2 -1.5 -1 -0.5 0 0.5 1 1.5 2

Time (s)
Figure 4-9

a.

Rspos2+ Proportion of Cell Activity

N = 81

85%

10%

5%

Inter-Trial  Cue  Non-specific
Figure 4-9

b. Whole Amygdala Proportion of Cell Activity

N = 111

- Correct Choice: 89%
- Cue: 9%
- Non-specific: 2%

![Pie chart showing the proportion of cell activity in the whole amygdala with N=111.]
4.7 Figure Legends

Figure 4-1: In-vivo Imaging strategy

A strategy for using the calcium indicator GCaMP6f to image ROIs in-vivo. (a) Diagram of behavior setup containing three pokes where odors and water rewards are delivered. A miniature endoscope is implanted with a prism lens that extends to the amygdala. The animal is trained to perform while wearing the mounted microscope. (b) Schematic of a trial. Activation of the odor port (center port) for a threshold amount of time will initiate a trial. The animal will make a choice by poking into one of the two choice ports (left or right). On correct trials, a water reward is delivered with a small variable delay. On incorrect trials, the port lights turn off upon choice port entry (error feedback). Blue boxes indicate the time window which is analyzed (=/- 2 seconds) although the entire session is recorded. (c) GCaMP6f was delivered into Rspo2+ neurons using an AAV-rh8 serotyped virus containing a CRE-dependent DIO construct. (d) Diagram of basal nucleus of the amygdala, where expression is constrained by the CRE-LoxP system.

Figure 4-2: Example frames from an imaging session

(a-d) Still images from a movie of an imaging session. The four frames represent four distinct points in time, in which different ROIs are active. Active ROIs are
circumscribed in white squares. The normalized difference in luminance above a threshold was used to calculate active events. (e) A sample trace representing the normalized difference in luminance for an example ROI. Deflections that pass a threshold (+2 SD from the mean) are counted as an event.

**Figure 4-3: Rspo2+ cue-inhibited ROIs**

One subset of Rspo2+ ROIs is inhibited by presentation of an odor cue. (a) an example raster plot for a ROI that is inhibited by cue presentation. Each tick represents a single event above threshold. Red line indicates the time of cue presentation. The occurrence of events greatly decreases after the presentation of the cue. (b) The average event rate across trials for the ROI in panel “a”, showing that event rate decreases upon cue presentation. (c) A second example Rspo2+ ROI which is inhibited by cue presentation. Inhibition occurs for a little over 0.5 seconds and then returns to baseline firing rate in this neuron. (d) a third example ROI in which inhibition occurs for a period of around 0.5 seconds after the onset of cue presentation. (e) An average across all Rspo2+ ROIs of this type showing that the peak of inhibition occurs shortly after the onset of the cue presentation, before returning to baseline. (f) The average activity for cue-inhibited ROIs has been subset into trials which were preceded by either a correct or error trial. Using an ANOVA we
cannot detect any difference in activity between these two subsets, suggesting that these neurons are not modulated by prior trials.

**Figure 4-4: Rspo2+ Inter-trial active ROIs**

A subset of Rspo2+ ROIs is activated during the inter-trial period. (a) an example raster plot for a Rspo2+ ROI that shows an increase in activity around 0.5 seconds after the presentation of a reward and the start of the following trial. (b) average spike rate for the ROI in the previous panel. (c) Average spike rate for all ROIs that are active in the inter-trial period, subset by the outcome of the previous trial. In both cases, activity peaks after the delivery of a water reward, however, activity is greater in cases where the previous choice was correct, than when it was an error.

**Figure 4-5: In-Vivo Imaging Strategy for Amygdala ROIs**

(a) Imaging all putative neurons in the amygdala required using a virus that is constitutively active in all neurons. We used an AAV viral construct that expressed GCaMP6f under the control of the human synapsin promoter (hsyn). (b) The virus was then injected unilaterally into the amygdala.

**Figure 4-6: Whole amygdala ROI cue response**
A subset of ROIs in the amygdala is activated by cue presentation. (a) raster plot of an example ROI in the amygdala that increases activity after presentation of a reward-predictive cue. Red line indicates the timing of cue presentation. (b) Average activity for the example ROI in previous panel showing a sharp increase in activity after the presentation of a reward-predictive cue.

**Figure 4-7: Whole amygdala ROI correct choice response**

A subset of amygdala ROIs is activated when the animal makes a correct choice. (a) An example raster plot of a ROI that increases its event rate after positive feedback from a correct choice. (b) The average event rate of the example ROI in the previous panel, showing an increase in activity upon correct choices. (c) Average event rate of all amygdala ROIs that were active upon entering the correct choice port.

**Figure 4-8: Whole amygdala ROI correct choice activity previous choice bias**

Bias can be detected in the neuronal firing pattern of individual ROIs. (a-b) two raster plots aligned to a correct choice shows that when activity is partitioned by the animal's previous choice, there is a difference in activity depending on whether the previous choice was correct or incorrect. The density of events is higher on trials in which previous choices were correct. (c) the average activity of all amygdala ROIs that
are responsive to correct choices, partitioned by the outcome of the previous trial (n-1 correct/n-1 error). An ANOVA shows a significant effect of the previous outcome, suggesting that correct choice activity is modulated by previous outcomes.

**Figure 4-9: ROI proportions**

In both Rspo2+ and pan-neuronal labeling groups, the majority of ROIs visualized did not have activity that aligned with any of the task events. These are ROIs in which events were detected but were not aligned to any of the task events. (a) A small proportion of ROIs in the Rspo2+ group responded either during the inter-trial interval or to the cue. (b) Similarly, in the pan-neuronal group, a small proportion of ROIs responded to correct choices and to the cues.