Protocol for Mice Behavioral Analysis in Response to Predator Cues

Sunaina Santhiveeran[1] and Sachiko Haga-Yamanaka[1]

1 Department of Molecular, Cell and Systems Biology

ABSTRACT

Mice exhibit defensive behaviors in response to various predator cues. When a mouse "senses" a predator at a close distance, it exhibits freezing behavior. Alternatively, when it senses bodily excretions from a predator, it escapes from the area. These behaviors are evolutionary responses to predators that help their increase survival. How animals sense the different types of predator-derived cues and induce appropriate behaviors in response to the specific predator cues have largely remained elusive.

In this study, we aimed to establish a method to analyze mouse behavioral responses toward various forms of predator-derived biological samples, such as cat saliva, which contain chemical cues. We categorized mouse responses to predator cue exposure as freezing, fear assessment, or exploratory behavior, each of which is triggered by different levels of fear that the animal is experiencing. The behaviors were quantified manually and compared between the animals exposed to control and predator-cue stimuli. We show that this protocol is effective in analyzing levels of fear in mice as there is a significant increase in the occurrence of fear-based behaviors in mice exposed to cat saliva.

Developing a strong protocol for quantifying fear-related behaviors is essential to understand brain mechanisms underlying behavioral responses induced by different types of predator cues in mice. Moreover, the present protocol can be further utilized to understand how different levels of fear are processed in an animal's brain circuitry.

FACULTY MENTOR

Dr. Sachiko Haga-Yamanaka

Assistant Professor in the Department of Molecular, Cell and Systems Biology

Dr. Sachiko Haga-Yamanaka is an Assistant Professor in the Department of Molecular, Cell and Systems Biology. Dr. Haga-Yamanaka received her PhD from the University of Tokyo. Dr. Haga-Yamanaka has focused on the mouse vomeronasal system that detects and discriminates specific molecular cues provided as pheromones and predator cues. These chemical cues trigger a repertoire of innate behaviors, including mating and defensive behaviors. Interestingly, brain neurons in the vomeronasal circuitry express a variety of hormone receptors, suggesting a potential mechanistic link between the vomeronasal circuitry and endocrine hormones. Using an *in vitro* cell culture system and *in vivo* mouse models, she aims to elucidate how the brain controls behavior through integration of external sensory signals and internal hormonal state.

Sunaina Santhiveeran

Department of Molecular, Cell and Systems Biology

Sunaina Santhiveeran is a fourth year Neuroscience major. Her research with Dr. Sachiko Haga-Yamanaka focuses on creating behavioral protocols to be used to uncover the mechanisms behind the defensive behavior exhibited by mice exposed to predator samples. Other notable experiences throughout her undergraduate career include working with large data sets as a UCR FIELDS intern at Caltech/JPL and volunteering in various capacities in the healthcare field. Her future plans are to combine these past experiences and pursue graduate studies in Translational Medicine in order to help increase access to quality healthcare through technology. Her work with Dr. Sachiko Haga-Yamanaka is supported by the University Honors Program and the Undergraduate Research Mini-Grant.

INTRODUCTION

Animals make behavioral decisions in response to external stimuli. For example, most prey animals have the ability to perceive the presence of a predator and exhibit defensive behaviors to increase the likelihood of their survival. When a prey animal "senses" a predator at close distance, it exhibits freezing behavior in order to disappear from the predator's vision. Freezing behavior is defined by a lack of movement for several seconds, allowing prey to disappear from the predator's view. Alternatively, when prey senses bodily excretions from a predator, it escapes from the area. This fleeing behavior is exhibited when the predator is in the area. These behaviors are defensive behaviors, practiced to escape predator detection or capture. Laboratory mice exhibit these defensive behaviors and other behavioral responses, such as paying attention solely to the predator cue while maintaining movement, approaching the cue in a hesitant manner, hiding in bedding, and examining the cue by extending their head and neck towards it while keeping their body away. These are stereotypic behavioral responses of prey animals practiced in order to maximize the chance of survival (Blanchard et al. 2001).

Prey rodents such as mice are able to perceive the presence of a predator by sensing chemical cues emitted from predators (Apfelbach et al. 2005; Osada et al. 2015.), which are detected by the main and accessory olfactory systems (Papes et al., 2010; Dewan et. al., 2013.). Interestingly, even inbred rodents, which have been isolated in the laboratory from other species for hundreds of generations, are known to respond to predator-derived cues and exhibit fear-like defensive behaviors to chemical cues contained in the saliva, urine, and feces of predators (Apfelbach et al. 2015.). This innate response suggests that the neural mechanism underlying this behavioral decision is genetically determined.

In order to understand this genetically-determined neural mechanism, in this study, we aimed to establish a standard method to analyze mouse behavior responses towards various forms of predator-derived biological samples containing chemical cues. For this purpose, we established three critical components of the analysis: a behavioral assay system, behavioral analysis platform, and mouse defensive behavior categorization. In the post hoc analysis, mouse behavior responses were categorized based on types of behaviors, types of exploratory sniffing, direction facing, and relative locations of the mouse.

By using this method, we found a significant increase in fear-related behavior responses, such as freezing, when mice were exposed to certain predator samples as compared to controls, indicating this method can be used for future behavioral analyses of predator defensive behavior in mice. Developing a strong protocol for quantifying fear-related defensive behavior is essential for understanding the neural mechanisms underlying the behavioral responses induced by different types of predator cues. Moreover, the present protocol can be further utilized to understand how different levels of fear are processed in an animal's brain circuitry.

METHODS

Habituation

Mice were habituated for three days before the recorded trial. Habituation was conducted by transporting the subject's home cage to the recording room and keeping them there for an hour after the experimenter leaves.

Behavior Assay

The recorded trial was conducted at the same time as the habituations. Before transporting the mice to the recording room, the food and water bottle were removed and the lids were replaced with flat lids. The bedding was also removed to eliminate all visual obstructions. Upon transportation into the recording room, the mice were habituated for an hour. The experimenter entered the room after the hour and placed the sample after ten minutes of allowing the mice to habituate to their presence. Once the sample was placed, the experimenter remained in the room for the duration of the video recording.

Samples were collected from domestic cats usually within 24 hours of each trial. These samples were either fur clippings or saliva swabs. A control was also conducted by placing a plain cotton swab in the home cage. The forceps were clean before each use.

Recording the Mice Videos

Videos were recorded using infrared lights and a night vision camera during the dark cycle. The videos were

Behavior	Description
Interacting	Mouse touches/plays with the stimulus. At the minimum, this is observed with the mouse prodding the sample with their nose and can also include the mouse actively playing with the sample. With cotton swabs, this often means the mouse bites it or chews it. With the fur samples, mice typically move it around or hold it.
Attending	Mouse stays still with head facing towards the stimulus, directing all attention to it. This can be differentiated from freezing behavior because there is still movement with the mouse, often actively sniffing towards the sample or moving towards or away from the sample. Typically, attending behavior is most often observed during the time of sample placement. This class of behavior is not frequently observed as it is an intermediate between freezing and searching + object sniffing.
Approaching	Mouse heads in a straight line towards the stimulus. This behavior is often accompanied with stretched sniffing. Mice often take roundabout routes along the walls of their enclosure or other ways to avoid the sample when they first approach it. Approaching behavior differentiates these more fearful approaches with a direct, exploratory approach. This is an exploratory behavior.
Hiding	Mouse burrows self in bedding. Not all mice tested were housed with enough bedding to burrow themselves in so this behavior was rare. This is an indication of fear.
Freezing	Mouse is still or shows minimal movement. Exceptions are respiration and movements associated with sniffing that last less than roughly a one second. This is the highest indication of fear.
Immobile	Mouse is still due to freezing or sleeping. This category exists for instances when the mouse's behavior is inconclusive, normally due to them facing away from the camera.
Sleeping	Mouse is still, with their body relatively curled up, their eyes closed, exhibiting respiratory movements as well as occasional twitching. This is a relaxed behavior and normally indicates a low level of fear.
Digging	Mouse digs through the bedding in the enclosure. This is an exploratory behavior and normally indicates a low level of fear.
Grooming	Mouse licks and cleans itself. This is a relaxed behavior and normally indicates a low level of fear.
Eating	Mouse eats, nibbles, or chews on something. This behavior category is not used if the object being gnawed is the sample; that would be under "interacting." Mice aren't provided any food during recording so it's often bedding or excrement that is consumed. This behavior category is rare but indicates a low level of fear as it is a relaxed behavior.
Rearing	Mouse stands on their back legs. This is typically an exploratory behavior and normally indicates a low level of fear. When exploratory, the mouse faces upwards towards the top of the enclosure and often grasps on the walls or the ceiling grid of the enclosure. Rearing can also be accompanied by freezing behavior when the mouse freezes in an upright position.
Searching	This is a default behavior. It typically involves the mouse wandering their environment but is used anytime the mouse is not performing any of the other defined behaviors. This is an exploratory behavior and normally indicates a low level of fear.
Undirected sniffing	This is a default behavior. It typically involves the mouse wandering their environment but is used anytime the mouse is not performing any of the other defined sniffing behaviors. This is an exploratory behavior and normally indicates a low level of fear.
Stretched sniffing	Mouse sniffs and stretches forward at the neck, holding their body back, in order to get closer to the sample. This is a fear assessment behavior and normally indicates a moderate level of fear.
Object sniffing	Mouse sniffs the object at close proximity. If the mouse stretches to sniff the object better, the behavior is then coded as "stretched sniffing." This particular form of object sniffing involves the mouse being close enough to touch the object. This form of object sniffing always accompanies the "interacting" behavior. This is a fear assessment or exploratory behavior and normally indicates a low to moderate level of fear.
Object sniffing (far)	Mouse sniffs towards the object from a distance in this form of object sniffing. This particular form is a fear assessment behavior and normally indicates a moderate to high level of fear.
Middle	Mouse is in the center of the enclosure with no part of their body (except for the tail) touching the walls. This category helps determine location and is based on the fact that samples were typically placed away from the walls of the enclosure. This is either a fear assessment or exploratory behavior.
Corners	Mouse is along the walls of the enclosure with some sort of physical contact with the walls (except for the tail). This category helps determine location and relates to higher level of fears since fearful mice were observed to hug the walls when navigating the enclosure or freezing during fear assessment or exploratory behaviors.
Towards sample	Used to better describe any of the previous behaviors. Mouse is facing the sample. When in combination with searching, typically means the mouse is moving in the direction of the sample. Cannot be used alone to determine level of fear.
Away from sample	Used to better describe any of the previous behaviors. Mouse is facing away from the sample. When in combination with searching, typically means the mouse is moving away from the sample. Cannot be used alone to determine level of fear.

Table 1. *Description of the behaviors coded into BORIS.*

recorded for roughly five minutes before sample placement and fifty-five minutes after placement.

Behavior Categorization

Table 1 details the categories used to quantify behaviors. These categories were determined upon watching the reactions of mice when exposed to cat saliva and based on the ethogram presented by Dr. Joseph Garner's lab at the Stanford School of Medicine (Garner et. al.). The behaviors were then organized into subcategories for fear behaviors, fear assessment behaviors, exploratory behaviors, location, and relaxed behaviors, based on the subcategories outlined by Blanchard et. al. (Blanchard et. al., 2003).

Fear behaviors include freezing and hiding. Freezing was determined to be a complete lack of movement, excluding sniffing movements that lasted less than two seconds. Hiding was only observed when mice had bedding to burrow in. Fleeing behavior was not observed in this experiment due to space constraints of the cages.

Fear assessment includes stretched sniffing, approaching, and attending. These behaviors indicate a level of caution and involve the mouse keeping their body at a distance while extending their head and neck towards the sample.

Exploratory behaviors were determined to encompass object sniffing, interacting, rearing, undirected sniffing, and searching. Undirected sniffing and searching were set to be the default behaviors. They are used to describe basic exploratory behaviors when no other specific behavior was observed. Exploratory behaviors are driven more by curiosity than fear.

Relaxed behaviors were performed when the mouse is feeling little to no fear and includes grooming, sleeping, and eating. Such behaviors are typically only performed when the mouse feels safe in their environment.

Location was observed to correlate with fear behavior when estimated as either middle or corners. Since videos were only recorded laterally, this was estimated based on

Behavior code	Excluded behaviors
freezing	hiding, approaching, interacting, searching, eating, grooming, sleeping, digging
hiding	freezing, approaching, interacting, rearing, searching, eating, grooming, sleeping, digging
stretched sniffing	undirected sniffing, object sniffing
undirected sniffing	stretched sniffing, object sniffing
object sniffing	stretched sniffing, undirected sniffing
attending	approaching, interacting, searching, eating, grooming, sleeping, digging
approaching	freezing, hiding, attending, interacting, rearing, searching, eating, grooming, sleeping, digging
interacting	freezing, hiding, attending, approaching, rearing, searching, eating, grooming, sleeping, digging
rearing	hiding, approaching, interacting, searching, eating, grooming, sleeping, digging
searching	freezing, hiding, attending, approaching, interacting, rearing, eating, grooming, sleeping, digging
middle	corners
corners	middle
eating	freezing, hiding, attending, approaching, interacting, rearing, searching, grooming, sleeping, digging
grooming	freezing, hiding, attending, approaching, interacting, rearing, searching, eating, sleeping, digging
sleeping	freezing, hiding, attending, approaching, interacting, rearing, searching, eating, grooming, digging
digging	freezing, hiding, attending, approaching, interacting, rearing, searching, eating, grooming, sleeping
towards sample	away from sample
away from sample	towards sample

Table 2. *Description of the behavioral exclusions coded into BORIS.*

whether or not the mouse's body (not including the tail) was touching the walls of the enclosure. Samples were usually dropped in the middle of the cage, so mice remaining in corners are likely indicators of caution or fear.

Combining these four types of behavior, general behaviors, sniffing behaviors, location, and direction facing, gives us a good idea of the level of fear the subject is experiencing. For example, a mouse exhibiting relaxed behaviors like grooming or eating in the middle of the cage exhibits low levels of fear. A mouse keeping to the corners and displaying defensive behaviors like stretched sniffing is experiencing more fear.

Behavior Coding

Coding originally began five minutes before sample placement to an hour after placement. Observations of initial trials/videos showed that most fear behavior occurred within 20 minutes after sample placement, so future coding was conducted from two minutes before sample placement to 20 minutes after sample placement. This seems to have been adequate, as fear behavior tended to decrease over time exposed. All coding was conducted using BORIS Behavioral Analysis software (Friard et. al., 2016).

BORIS is a software that allows users to define their own behaviors and then manually code them to either a live video or a prerecorded video. Behaviors can also be grouped into categories or given modifiers to better describe events. Users can also define their own independent variables as well. The program is also capable of conducting basic statistical analysis, including providing average durations for observed behaviors and the export of the data in Excel form.

This feature was utilized to omit behaviors that would be exclusive to one another, ensuring that at any given point of the coding, there is only one of each type of behavior being performed. The exclusions programmed into BORIS are featured in ***Table 2***.

RESULTS

A total of thirty videos were analyzed using this protocol. The coding was analyzed by looking at the length of time each behavior was coded for over the twenty minutes that were analyzed. BORIS also provides raster plots of the behaviors coded for each video. ***Figure 1*** shows a raster plot for both a control and a mouse exposed to cat saliva. These can be used to visualize the behaviors observed.
A comparison of the data collected for three controls and three trials of cat saliva exposure can be found in the bar graphs in ***Figure 2a.*** ***Figure 2a*** shows that mice exposed to the cat saliva exhibited significantly less object sniffing and significantly more undirected sniffing. Object sniffing from afar and stretched sniffing were not significantly different between the control and saliva-exposed mice. ***Figure 2b*** shows that there is no significant difference in the direction facing between control mice and saliva-exposed mice, though there is a tendency for control mice to face towards the sample more often than away whereas saliva-exposed mice tended to face both ways for similar amounts of time. In ***Figure 2c,*** while there is no significant difference in the behaviors of the control mice and saliva-exposed mice, there was a tendency for the control mice to remain in the middle of the cage longer compared to trial mice, of which tended to stay near the corners. ***Figure 2d*** shows significant increase in freezing behavior and a significant decrease in rearing behaviors and interacting in the cat saliva exposed mice. There is also a noticeable decrease in relaxed behaviors, including grooming and digging, that was observed in control mice.

DISCUSSION

Using this method, we found a few significant differences between the control and the saliva-exposure trials. The mice exposed to the control swabs interacted with it for far longer than the mouse with the sample swab, indicating low levels of fear. There was also a significant increase in freezing behavior expressed with the cat saliva that wasn't expressed with the control, indicating a high level of fear. This matched expectations that there would be increased fear in mice presented with predatory cues. There was a less significant difference in the direction facing behaviors between the two groups, indicating that this category may not be necessary in future analysis. Although there was a trend for control mice to stay within the middle of the cage while saliva-exposed mice stay near the corners, this difference was not significant and may need further specification. For example, an overhead recording would allow for a more specific analysis of distance from the predator sample. The location and direction facing values could, in addition, be used in conjunction with the other behaviors to describe them better.

Overall, the method presented proved useful for analyzing behavior in response to predator cues and would be well-suited for analyzing fear responses to other stimuli as well. The results follows expectations of increased fear behavior in response to predator cues, ensuring that it is a fit method for future fear-behavior analysis.

CONCLUSION

This method provides a foundation for analysis of mouse defensive behavior responses to predator cues. The significant increase observed in fear behaviors catalogued using this method of analysis matches expectations that mice react defensively in response to cat chemical cues. This method could be further combined with neural recording and neural manipulation techniques to uncover the brain regions associated with the perception of fear. Revealing these underlying neural mechanisms is not only significant in elucidating how sensory signals are processed to trigger behavior, but also in understanding the brain mechanisms of fear and anxiety in humans.

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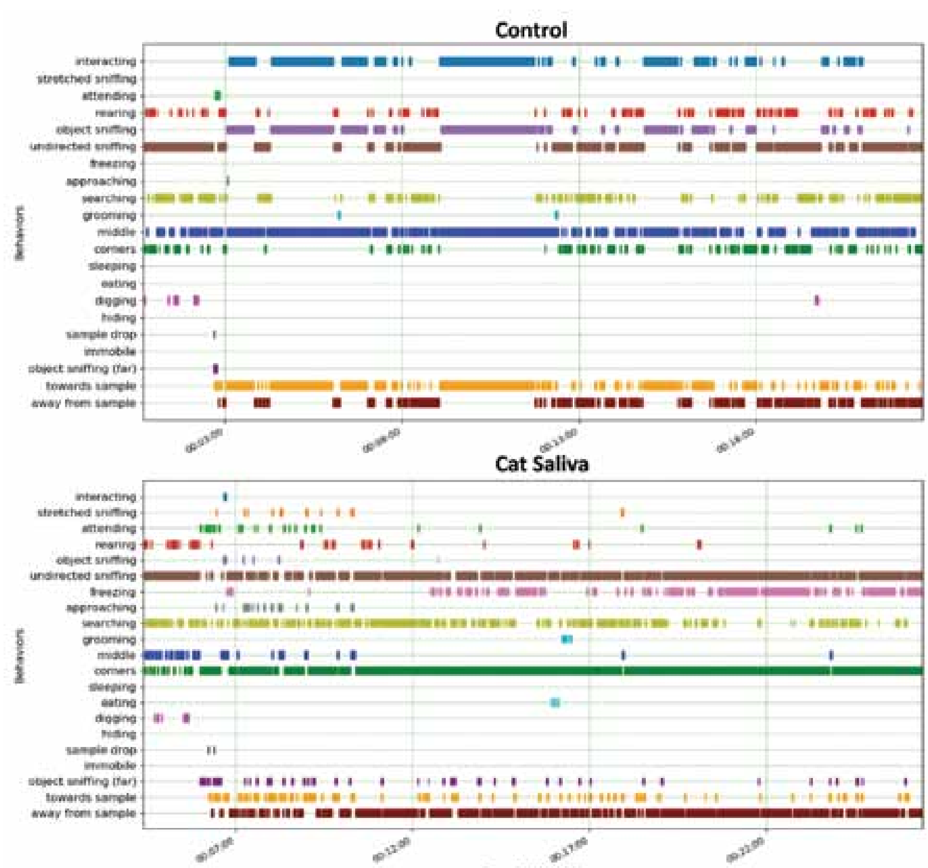

Figure 1. *Raster plots of mouse behavior in control mice (top) and cat saliva exposed mice. (bottom). Differences to note include: the decreased interaction with the saliva sample, the lack of stretched sniffing with the control, the increase in object sniffing with the control, the complete lack of freezing behavior in the control, the decreased incidence of approaching with the control, the increased time spent in corners than the middle with the saliva sample, and the increase in object sniffing (far) with the control.*

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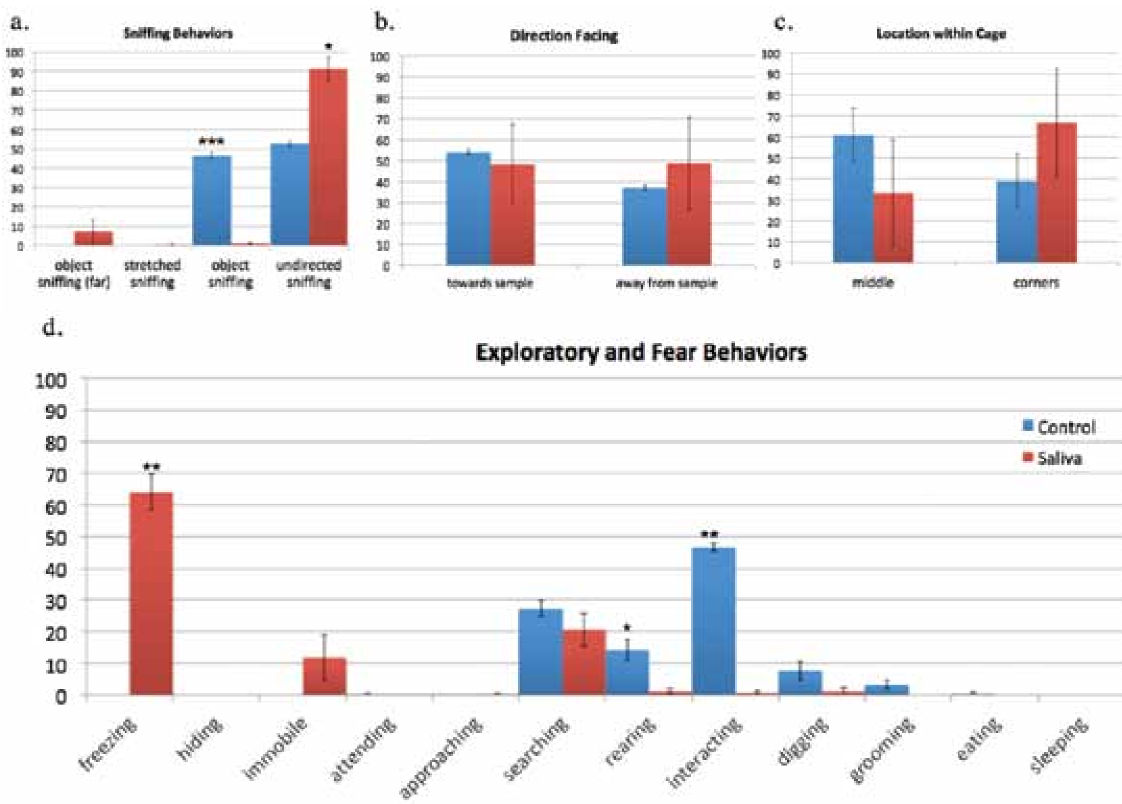

Figure 2. *Bar graphs displaying percent of total time of mouse behaviors. Values are averages ± SEM; n= 3 mice. Significance was determined using a two-tailed T test with 2 degrees of freedom.* ***Figure 2a*** *shows a significant difference in object sniffing and undirected sniffing.* ***Figure 2b*** *compares the incidence of the direction facing for each trial type. There is no significant difference for either but a trend can be seen with the control mice exhibiting a tendency to face towards from the sample.* ***Figure 2c*** *compares the location of mice in both trials. There is no significant difference for either but a trend can be seen with the control mice exhibiting a tendency to stay in the middle of the sample and vice versa for the saliva trial mice.* ***Figure 2d*** *shows a significant increase in freezing behavior and a significant decrease in interacting and rearing behaviors with the saliva exposed mice. There is also a non-significant but noticeable decrease in digging and grooming behaviors in the exposed mice as well.*