Roles of Retinal Circuits in the Innate Visual Behaviors of Mice

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Abstract

Much of brain science is concerned with understanding the neural circuits that underlie specific behaviors. While the mouse has become a favorite experimental subject, the behaviors of this species are still poorly explored, posing a challenge for understanding the neural basis for visual behaviors. We approach this problem first, by exploring the visual responses of mice of mice to visual stimuli, second by genetically ablating specific cell types in the retina and testing the effects on visual behaviors. We show that mice respond to the visual display of an approaching object by either initiating escape or by freezing for an extended period. We then use genetic means to ablate specific cell classes in the retina to dissect the roles of these classes in the looming avoidance behavior and two other visual behaviors: optokinetic reflex (OKR) and pupillary light reflex (PLR). We show that a class of cells including the alpha retinal ganglion cells are necessary for the looming avoidance behavior, while this class has minor roles in the pupillary constriction and optokinetic reflex. By contrast another cell class, starburst amacrine cells, are crucial for the optokinetic reflex and are dispensable for the looming avoidance and pupil reflexes. Our results suggest that mouse retina possesses neural circuits that are specialized for specific visual functions, such as avoiding an aerial predator, moving head to minimize retinal slip and constricting the pupil to limit light input to the retina.

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Introduction

One of the main goals of neuroscience is to understand how organisms use sensory information to produce behaviors that support their survival. A common principle across sensory systems is *feature detection*: the ability of the sensory neurons to signal a specific feature of the external stimulus while ignoring others. Often, neurons that detect distinct features can be identified by their distinct morphology or unique expression of a molecular marker. In the olfactory and gustatory systems the receptor identity of the sensory neuron determines this feature specificity (Vosshall et al., 2000; Chandrashekar et al., 2006; Yarmolinsky et al., 2009). In the vertebrate visual system, morphologically distinct retinal ganglion cell types detect different visual features (Masland, 2012). Physiological measurements from different sensory neurons reveal that defining the "feature" that the neuron is selective for is not straight-forward for all neurons. While some neurons respond to a well defined single feature, may respond to a range of stimuli and the same feature can activate multiple types of neurons (Wilson, 2013). Even highly specialized neurons might project their axons to higher order neurons that are have wider tuning that the receptor neuron (Schlief and Wilson, 2007). Moreover the feature selectivity could change depending on the internal state of the animal (Chao et al., 2004). What is the benefit of having a sensory neuron dedicated to the detection of a single stimulus feature? What could be the benefit of having a neuron that is more widely tuned or flexible? These questions cannot be addressed by analyzing the sensory system in isolation. They should be studied in the context of other constraints that could be put on the sensory system by the behavioral needs of the animal (Salinas,

2006). Since this will be different for each organism, a good understanding of the behavioral repertoire of the organism in question as well as tools to access its sensory system are essential to link the sensory processing to the behavioral outputs.

In the studies of the visual system, the mouse has become a popular organism, due to the genetic accessibility of different neural types. However the knowledge on its natural behaviors is still relatively scarce, which is a road block to understanding the behavioral relevance of these neurons. The purpose of this thesis is (1) to describe a novel visual behavior we discovered in the mouse, (2) our initial efforts to link the roles of two retinal cell types to this and other innate behaviors. Ultimately we would like to understand if and how specialized particular cell types are for particular behaviors in the mouse and how this serves the behavioral needs of the animal. Examples from other organisms and other sensory systems give us clues about the potential benefits and drawbacks of having cell types tightly linked to behavior. The first part of the introduction will discuss these examples. A second part will follow with a more detailed discussion of the mammalian retina and discuss the role of feature detection in the mouse retina.

Dedicated sensory neurons for innate behaviors

The causal relationship of a neural circuit and a particular behavior can be established by ablating the neurons of interest and measuring the effects on behavior. Such studies have revealed that some sensory neurons are dedicated to specific innate behaviors. These neurons often detect stimuli that have critical value for the animal's survival such

as predators or food sources. Laser ablation studies in the earthworm *Caenorhabditis elegans* revealed that sensory neurons AWA and AWC are critical for chemotaxis behavior towards volatile odorants but not for water-soluble odorants (Bargmann et al., 1993). By contrast a distinct set of sensory neurons are required for chemotaxis towards water-soluble attractants (Bargmann and Horvitz, 1991). Water soluble attractants like salt could have critical functions like maintaining electrolyte homeostasis for the animal, while volatile odorants signal food and could help with energy metabolism. A distinction at the sensory level at this level ensures that the animal is able to distinguish the two stimuli from each other, just like mammals process taste and odors separately. Another sensory neuron AWB mediates avoidance response to a particular odorant 2-nonanone, a common solvent in pesticides, in this organism (Troemel et al., 1997).

Similarly in the fruit fly *Drosophila melanogaster*, distinct sets of olfactory receptor neurons mediate attraction to appetitive odors that signal potential food sources or pheromones. (Min et al., 2013; Semmelhack and Wang, 2009; Schlief and Wilson, 2007). On the other hand a single olfactory receptor neuron population is dedicated to sensing and mediating avoidance to CO^2, a gas emitted from stressed flies (Suh et al., 2004). Receptor neurons that are sensitive to water and are necessary for water seeking behavior have also been identified in labellum of this organism (Inoshita and Tanimura, 2006).

Examples of sensory systems dedicated to essential approach or avoidance have also been identified in mice. Similar to the fruit fly, the mouse has an olfactory subsystem dedicated to CO^2 aversion (Hu et al., 2007). It has been shown that the dorsal olfactory bulb is dedicated for innate olfactory avoidance behaviors (Kobayakawa et al., 2007). While animals are capable of sensing aversive odors in the absence of the dorsal glomeruli, they no longer avoid them. A recently discovered olfactory subsystem called Grueneberg ganglion cells were shown to respond specifically to alarm pheromones and mediate avoidance behavior (Brechbuhl et al., 2008; Stowers and Logan, 2010). Two distinct sets of taste receptors both sensitive to salt mediate salt aversion and attraction behaviors (Oka et al., 2013).

Sensory neurons dedicated to approach or avoidance behaviors are not limited to olfactory and gustatory systems though those have been studied most heavily in this context. Neurons that are specialized “looming detectors” and trigger escape behaviors in response to approaching motion have been identified in the visual system of many species (de Vries and Clandinin, 2012; Fotowat and Gabbiani, 2011). In addition to signaling approaching predators, visual cues are also essential for detecting self motion, especially for organisms that are in constant motion like fruit flies or fish. Neurons dedicated to detecting global motion and optokinetic eye and body movements have been identified in the fruit fly as well as zebrafish and the mouse (Maisak et al., 2013; Portugues and Engert, 2009; Yonehara et al., 2009).

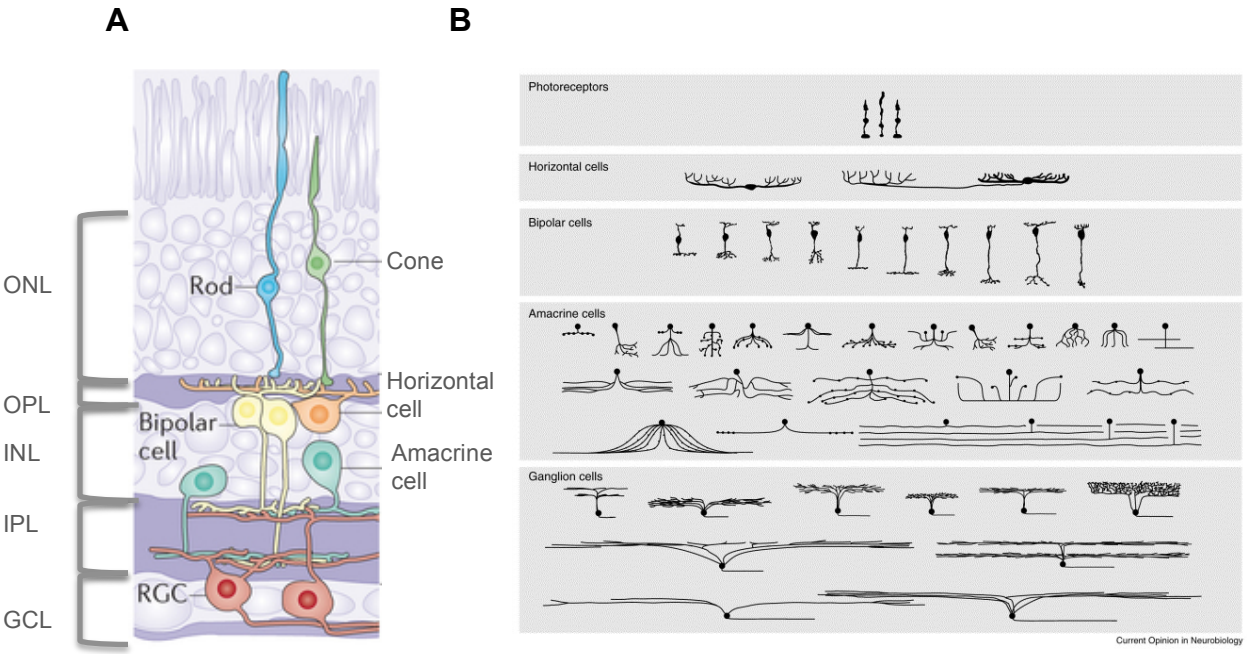
Flexibility of feature detection

While there are specialized sensory neurons dedicated for specific stimuli, there are also sensory neurons that respond to a larger variety of stimuli as well as sensory stimuli that stimulate many receptor neurons. As with dedicated neurons, it is useful to think about these in terms of the benefits to the animal. Having more than one receptor respond to the same stimulus feature might provide fidelity to the system (Bargmann and Horvitz, 1991). On the other hand environmental conditions that affect the internal state of the animal might make some stimuli more or less aversive requiring flexibility of the connection between the sensory input and motor output. For instance the worms show reduced aversion to a normally aversive odor when they are hungry and this is mediated by a switch in the receptor neurons that are activated differentially during hunger and satiety (Chao et al., 2004). The intensity of the stimulus is also important in addition to the qualitative properties. Activation of a dedicated pair of glomeruli is necessary to trigger attraction to low concentrations of vinegar, while at high concentrations this stimulus triggers repulsion via recruitment of an additional glomerulus (Semmelhack and Wang, 2009). Similarly in the mammalian system high salt conditions recruit neurons normally responsive to bitter and sour to mediate aversion (Oka et al., 2013). In the mammalian retina, the responses of the retinal ganglion cells change under different levels of ambient light (Tikidji-Hamburyan et al., 2015). How this affects visual behaviors of the mouse is an open question.

Mammalian retina and feature detection

The mammalian retina is one of the most heavily studied parts of the brain in terms of physiology, molecular identity and development of its cell types. It is made up of three cell body layers: outer nuclear (ONL), which hosts the photoreceptors, inner nuclear (INL), which hosts bipolar cells, horizontal cells, amacrine cells, and ganglion cell layer (GCL). The outer plexiform (OPL) is the synaptic layer where photoreceptors contact bipolar and horizontal cells, while the inner plexiform (INL), hosts connections between bipolar, amacrine and ganglion cells. Information flows from the photoreceptors to bipolar cells to retinal ganglion cells, which are the output neurons (Figure 1A). Horizontal cells and amacrine cells mediate lateral interaction of signals. The concentric spatial and biphasic temporal receptive field structure of most retinal ganglion cells (RGCs) gives the retina the properties of a spatial and temporal pre-filter of visual input (Kuffler, 1953). In addition the retina performs light adaptation and contrast adaptation, both of which ensure the encoding of visual features for a wide range of background illumination and contrast (reviewed in (Meister and Berry, 1999)). While these capabilities are useful for fast and reliable coding of visual information, they do not explain the purpose of the highly sophisticated anatomical circuitry of the mammalian retina. The mammalian retina contains at least 55 cell types with distinct morphologies and synaptic connectivity patterns (Masland, 2001). This anatomical diversity suggests that the retina is more than a simple pre-filter of visual input. One alternative hypothesis for the function of the retina is that it is comprised of parallel microcircuits each of which has evolved to encode specific features of the visual scene acting as a feature detector.

Figure 1: Diversity of cell types in the mammalian retina



(A) The mammalian retina has 5 major cell types. Signals from photoreceptors are transmitted to bipolar cells and then to retinal ganglion cells. Lateral connections are mediated by horizontal cells and amacrine cells. ONL = Outer nuclear layer, OPL = Outer plexiform layer, INL = Inner nuclear layer, IPL= Inner plexiform layer, GCL = Ganglion cell layer. (B) Each type has many subtypes that are morphologically and physiologically distinct. Most types are conserved between mammalian species. See (Masland, 2001; Euler et al., 2014; MacNeil and Masland, 1998) for detailed information on different types. (Image courtesy Euler et al., 2014 and Masland, 2001).

The idea of feature detection at the level of the retina originated from the pioneering work of early vision scientists and ethologists. Ethologists proposed the existence of neural mechanisms that trigger responses to only specific stimuli. They named such stimuli “sign stimuli” and mechanism that detect those “releasing mechanisms” (Zupanc, 2010). In the retina, discovery of ON and OFF cells that have classical center-surround receptive fields (Kuffler, 1953) was quickly followed by discovery of color specific cells in the monkey (Hubel and Wiesel, 1960), horizontal edge detector in the pigeon (Maturana and Frenk, 1963), direction selective units in the pigeon and rabbit retinae (Maturana and Frenk, 1963; Barlow and Hill, 1963), and non-linear “Y” units in the cat retina (Enroth-Cugell and Robson, 1966). Therefore it was clear to the vision scientists that retinal ganglion cells do more simple intensity calculations but instead respond to specific features of the stimuli. Horace Barlow, a pioneer in vision research, suggested that the “off” units in the posterior frog retina could serve as “fly detectors” since they are located on part of the retina where the image of a fly would fall (Barlow, 1953). Ethologist Niko Tinbergen advocated for the existence of microcircuits for feature detection while describing the gaping response of young thrushes. These animals display a gaping response towards moving objects only if they are above the horizontal plane through the nestling’s eye. As Tinbergen put it, “obviously this is a problem of the nervous system rather than that of the sense organ!” (Tinbergen, 1974). Since Barlow and Tinbergen more evidence has accumulated for retinal ganglion cell types that act as feature detectors.

Perhaps the most convincing evidence is the variety of retinal ganglion cell types in the mouse retina. Three distinct types of direction-selective ganglion cells (DSGCs) have been identified by electrophysiological studies: the ON and ON-OFF cells that report motion in four cardinal directions (Sun et al., 2006; Weng et al., 2005) and the OFF cells that respond to upward motion (Kim et al., 2008). More recent studies took advantage of the advanced mouse genetics and put molecular tags on different types of RGCs. This led to further characterization of existing cell types (Huberman et al., 2009) and identification of novel types. An approach detector circuit that contains PV-5 RGCs and is potentially involved in predator detection has been identified (Munch et al., 2009). Our laboratory in collaboration with the Sanes laboratory has identified several different cell types with distinct response properties. OFF JAM-B cells and ON-OFF BD cells respond selectively to upwardly moving objects whereas W3 cells do not have a direction preference but are responsive to very small moving objects (Kim et al., 2008; Kim et al., 2010; Zhang et al., 2012). These have also been described as "local edge detectors" in the rabbit retina (Levick, 1967; van Wyk et al., 2006). In fact, a parallel can be drawn between the asymmetric localization of W3 cells in the mouse retina and Barlow's "fly detector off units" in the frog retina (Barlow, 1953). W3 cells, like Barlow's "off units," are conveniently located to detect small flying objects such as faraway birds.

These discoveries present clear evidence that the retina hosts high level computations previously thought to be done by higher brain areas. What role do these highly specialized circuits have in the animal's behavior? Two types of RGC types have been linked to innate visual behaviors. The optokinetic response, the reflexive movement of the head and the eyes to track global motion, has been linked to the ON type direction

selective cells (Yonehara et al., 2009). The intrinsically photosensitive RGCs have been shown be necessary for entrainment of circadian rhythms and pupillary light reflex (Chen et al., 2011). What about the remaining ~ 20 RGC types? Almost nothing is known about their behavioral roles. As a first attempt to understand the behavioral roles of these cell types we hypothesize that each retinal circuit is dedicated to a specific innate visual behavior. The prediction from this hypothesis is that ablating one circuit will affect one behavior while sparing others. Testing this hypothesis requires genetic access to specific cell types and a repertoire of visual behaviors to test.

Mouse visual behaviors

Unfortunately the field of mouse visual behavior has been largely underexplored, perhaps due to the misconception that mice are non-visual animals. This view not only falls short of explaining the sophisticated neural circuitry of the mouse retina, but is also weakened by extensive evidence for visual mouse behavior. For instance, mice were shown to use primarily visual cues for formation and protection of territory from intruders (J.H, 1970; Strasser and Dixon, 1986). Deer mice have different orientation preferences under different illumination conditions, suggesting that vision is an important sensory modality for deer mice (Barry, 1982). Most tests of mouse visual behavior in the laboratory involve training paradigms where mice have to learn to detect simple visual cues. While this approach helps experimenters to have more control over the experimental parameters and obtain data more efficiently, it has two major shortcomings: (1) Almost all of the training paradigms use highly artificial stimuli such as

sinusoidal gratings that animals never encounter in the wild, (2) They involve training of the neural circuits for a specific task, therefore do not reveal the function of circuits under natural conditions. An alternative approach is to study innate behaviors that are presumably shaped and maintained by nature to aid the survival of the animals. Horace Barlow once stated (Barlow, 1961) *"A wing would be a most mystifying structure if one did not know that birds flew."* Just as it would be futile to understand all the parts of a wing without understanding its function in flight, it would be futile to study retinal circuits without an understanding of the animal's visual behavior in its natural setting.

Studies of innate responses in other organisms have made significant contributions to our understanding of principles of neural circuits. Some examples are prey localization of barn owls using auditory cues (Payne, 1971; Knudsen et al., 1977; Knudsen and Konishi, 1978) or visual prey and predator recognition in the toads (Ewert, 1974). In the mouse visual system such an approach has not yet been taken due to the scarcity of known of innate behaviors. Reflexive head and eye movements in response to global motion i.e the optokinetic response (Prusky et al., 2000), the natural aversion to bright light (Cryan and Holmes, 2005) and aversion to visual cliffs (Fox, 1965) are three behaviors that are commonly used in laboratories. Among these the optokinetic response has been linked to the ON type direction selective cells (Yonehara et al., 2009) and light aversion only requires a single photoreceptor to detect light levels. Therefore more behaviors are needed to understand the roles of ~20 retinal circuits (Masland, 2001).

In the first chapter of this thesis I present a novel innate visual behavior we discovered: looming avoidance response. Mice were found to exhibit robust flight and freezing responses to the visual display of an overhead looming stimulus mimicking an aerial predator. Chapter one includes descriptions of this behavior as well as internal and external factors that influence it. In the second chapter, I depict the experiments that aim to understand how different retinal cell types influence innate visual responses. We used the tools from mouse genetics that allowed us to kill specific cell types using toxins and measured behavioral defects. Specifically we focused on two retinal types: alpha cells and direction selective cells and their roles on three innate behaviors: looming avoidance, optokinetic reflex and pupillary light reflex. In our efforts to understand why mice use their vision, we also studied the air-righting reflex: mice right themselves up during a free fall to land with limbs pointing down. This reflex turned out to be independent of visual cues, however it is a useful behavior for future studies. Chapter 3 details the studies of this reflex.

The work presented in this thesis can be considered a first attempt at dissecting the neural circuitry of an innate visual behavior that has obvious ecological significance for the mouse. It also provides more insight into the specificity of each retinal cell type and its behavioral role. Killing of cells with toxins is crude but a useful first step to understand the essential elements of neural circuits. I hope that this work will inspire further studies that are more specific and less disruptive in nature to illuminate the beautiful dynamics of behavior and the sophisticated processes underlying them.

Chapter 1: Rapid innate defensive responses of mice to looming stimuli

Summary

Much of brain science is concerned with understanding the neural circuits that underlie specific behaviors. While the mouse has become a favorite experimental subject, the behaviors of this species are still poorly explored. For example, the mouse retina, like that of other mammals, contains ~20 different circuits that compute distinct features of the visual scene (Masland, 2012; Gollisch and Meister, 2010). By comparison, only a handful of innate visual behaviors are known in this species – the pupil reflex (Chen et al., 2011), phototaxis (Bourin and Hascoet, 2003), the optomotor response (Prusky et al., 2004), and the cliff response (Fox, 1965) – two of which are simple reflexes that require little visual processing. We explored the behavior of mice under a visual display that simulates an approaching object, which causes defensive reactions in some other species (Card, 2012; Yamamoto et al., 2003). We show that mice respond to this stimulus by either initiating escape within a second or by freezing for an extended period. The probability of these defensive behaviors is strongly dependent on the parameters of the visual stimulus, presence of a hiding place and mouse's previous exposure to the stimulus. Directed experiments identify candidate retinal circuits underlying the behavior and lead the way into detailed study of these neural pathways. This response is a new addition to the repertoire of innate defensive behaviors in the mouse that allows the detection and avoidance of aerial predators.

Results

For the mouse, avoidance of aerial predators, such as hawks and owls, is a central survival function, likely supported by dedicated brain circuits. The only useful sensory modality for this purpose is vision. Thus we searched for innate visual behaviors that would support defense from overhead threats.

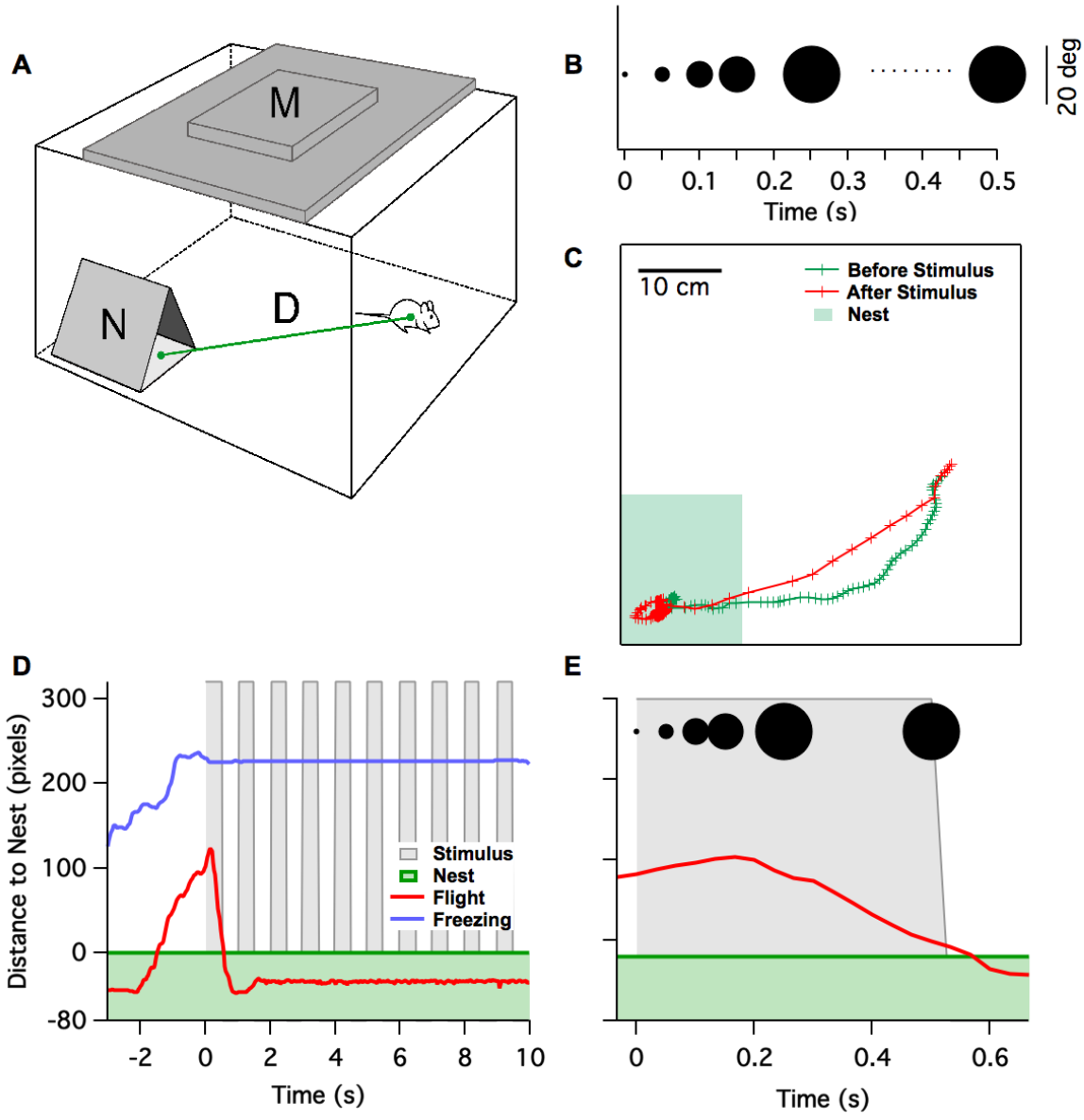
Visual display of an expanding dark disc triggers immediate flight or freezing while inhibiting rearing in mice

A wild-type mouse was placed into a behavioral arena with a display monitor covering most of the ceiling. An opaque nest in one corner of the arena offered a hiding place from visual stimuli (Figure 2A). The mouse was allowed 10 min of acclimation in the arena with a plain gray monitor. In this period the animal commonly displayed exploratory postures such as rearing on the hind legs and sniffing. Then the “looming stimulus” was started: On a gray background a black disc appeared directly above the animal at a diameter of 2 degrees of visual angle, expanded to 20 degrees in 250 ms, and remained at that size for 250 ms (Figure 2B). This stimulus was repeated 15 times with 500 ms pauses. This reliably triggered one of two behaviors: escape or freezing (Figure 2D).

Figure 2: A dark expanding disc in the upper visual field triggers flight and freezing.

(A) Schematic of the experimental setup: a box with a display monitor (M) on the ceiling and an opaque nest (N) in a corner. Multiple cameras monitor the animal's movements, from which one can measure the distance (D) to the nest. (B) Expansion of the looming stimulus in time from 2 degrees to 20 degrees. (C) An example trajectory of the mouse ~3 seconds before (green) and after (red) stimulus onset. The outline of the box shows the boundaries of the arena. Each tick is 33 ms. (D) Distance of the mouse from the nest before and during the stimulus. Example traces for flight (red) and freezing (purple) behaviors. Gray trace indicates the repetitions of the looming stimulus. See also Supplementary Movies 1 and 2. (E) Detail of the flight trace in (D) to emphasize the onset of the run to the nest in relationship to the disc expansion from 2 to 20 degrees.

Figure 2 (continued): Dark expanding disc in the upper visual field triggers flight and freezing

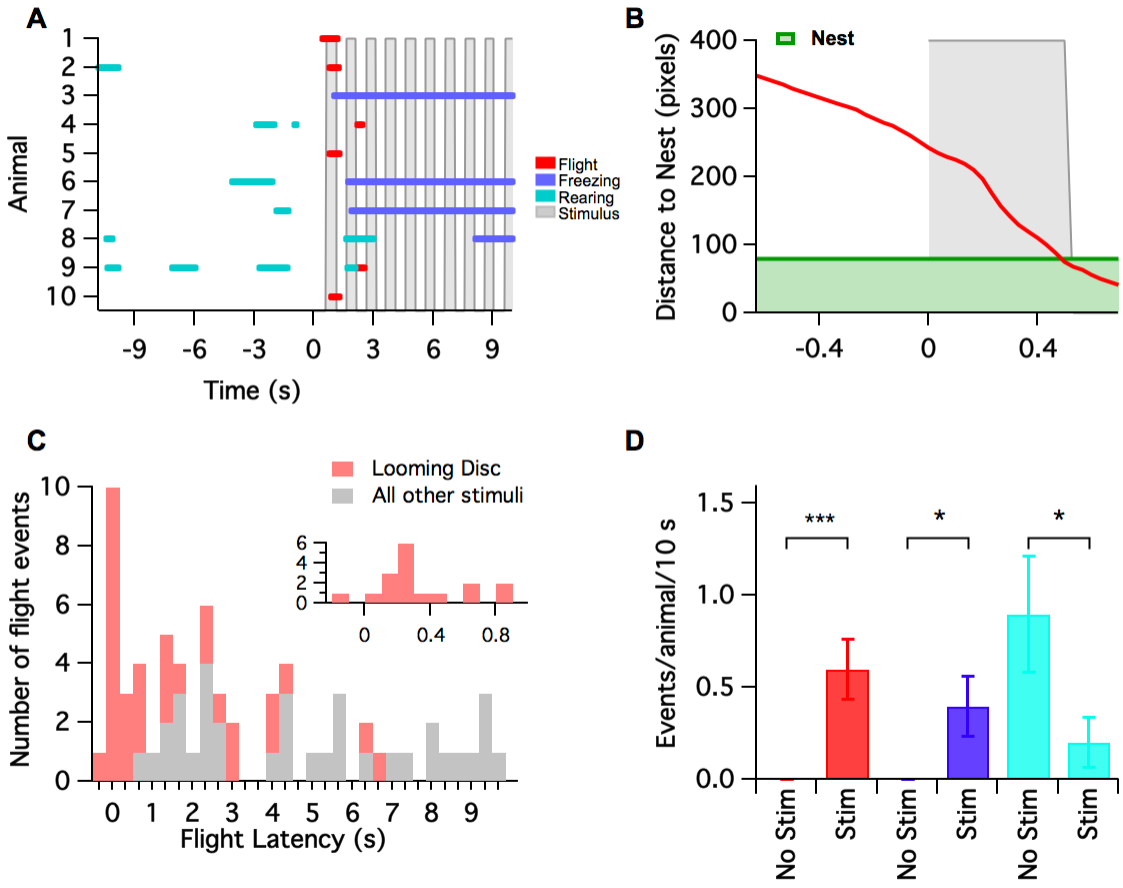


Most animals initiated a rapid escape to the nest (Figure 2 C-E; Figure 2A; $p < 0.005$; Supplementary Movie 1). Three of ten animals began their flight with a latency of less than 250 ms after stimulus onset, even before the disc reached its maximum size of 20 degrees (Figure 2E; Figure 3A). Such short-latency responses were observed repeatedly over many experiments (Figure 3C, Figure 4 A, I, J, K). In one case the animal had already initiated a run towards the nest prior to stimulus onset, but accelerated once the looming disc appeared (animal 1, Figure 3A, B). The animals that did not flee responded by freezing, often for the remaining duration of the stimulus (Figure 2D; Figure 3A, D; $p<0.02$; Supplementary Movie 2). The looming display also suppressed the animal's exploratory behavior, as observed by scoring the rearing events (Figure 3A, D; $p < 0.02$). Here we focus on the analysis of rapid escapes – with a latency below 1 s – and upward rearing events.

A looming dark disc is uniquely effective in driving sub-second flight and extended freezing behaviors

To investigate how different parameters of the looming stimulus influence the behavior, we tested five different stimulus conditions. First, when the same stimulus was presented in the lower visual field, with a display monitor below the floor, it caused no escapes or suppression of rearing (data not shown), suggesting that the looming response originates in the inferior retina. Stimulation from the top, but with a disc of reversed contrast (white on gray) produced no sub-second flight events (Figure 4B).

Figure 3: Statistics of reactions to the looming stimulus



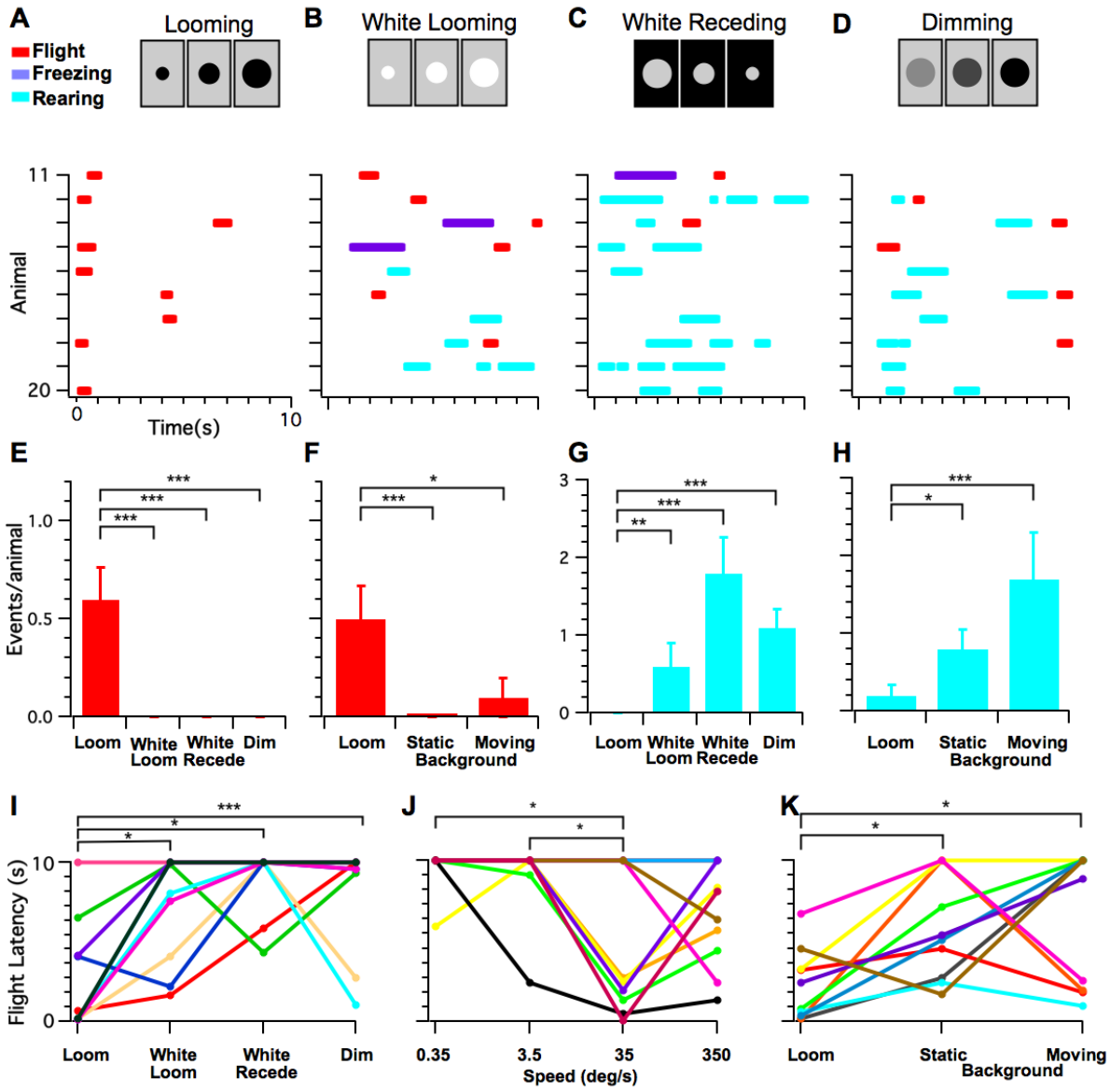
(A) Occurrences of flight, freezing, and upward rearing behaviors 10 s before and after stimulus onset. (B) Distance of mouse 1 to the nest before and after the stimulus. The speed increases more than 2-fold at 0.2 s after stimulus onset. (C) Histogram of flight latencies for looming stimuli (red) and all other stimuli tested (gray). The inset shows the distribution of sub-second flight events. (D) Probability of each of the three behaviors before and after stimulus onset. Error bars show standard error of the mean.

In the retina, the visual signal splits into ON and OFF channels that respond to a light increase and decrease respectively, and the above result points to a special role for the OFF channel in the looming response. However, a mere dimming of a disc of constant size, that matched the overall intensity change of the looming stimulus, failed to trigger rapid flight responses (Figure 4D) suggesting that motion of a dark edge is essential. To test whether dark edge motion is sufficient, we displayed a bright receding disc, which has dark edges that move inward rather than outward. This stimulus also failed to evoke a flight response in less than 1 s (Figure 4C). Under each of these alternative conditions, some animals did flee to the nest over an extended period of 10 s. However these events were less frequent and at much longer latency than under the dark expanding disc (Figure 4B-D, I). Furthermore, the dark expanding disc was the only condition that fully suppressed exploratory rearing, or produced prolonged freezing (Figure 4G). In summary the looming black disc is uniquely effective in triggering defensive responses in mice, even compared to closely related visual displays.

Figure 4: The frequency and speed of defensive behaviors depend strongly on stimulus parameters.

(A-D) Comparison of four stimulus displays: black looming disc (A), white looming disc (B), white receding disc (C), dimming disc (D). For each condition an ethogram indicates occurrence of three behaviors after stimulus onset at time 0: flight (red), freezing (purple), and upward rearing (cyan). The experimental sequence was B-C-D-A. (E) Frequency of sub-second flight in each of the four disc displays from panels a-d. (F) Frequency of sub-second flight after addition of a patterned background, either static or moving. (G-H) Frequency of upward rearing events under the stimulus conditions of panels E-F. (J-K) Flight latencies observed in 10 animals under the four disc displays (i), as a function of expansion rate of a dark disc (j), and as a function of background pattern (K). Each trace is from a different animal. Error bars show standard error of the mean.

Figure 4 (continued): The frequency and speed of defensive behaviors depend strongly on stimulus parameters



The speed of the expansion strongly influences the latency of flight

To explore visual stimulus space further, we took guidance from known retinal physiology. Several types of retinal ganglion cells (RGCs) are specialized for the detection of motion, but they differ in their tuning to motion velocity. The ON-DS cells are tuned to low speeds, ranging up to ~2 deg/s in the rabbit retina (Sivyer et al., 2010; Oyster, 1968), whereas the ON-OFF DS (Sivyer et al., 2010; Oyster, 1968; Weng et al., 2005) and the OFF DS (Kim et al., 2008) cells respond at much higher speeds. We explored the dependence of the flight behavior on the expansion speed of the dark disc, ranging from 0.35 to 350 deg/s. Sub-second flight events were observed only at 35 deg/s. A ten-fold higher speed was moderately effective, though with longer latencies (Figure 4J). Ten-fold lower speeds were ineffective. Therefore, if the ON-DS cells of the mouse resemble those of the rabbit in speed tuning, they are unlikely to drive the looming response, a conclusion reinforced by the weak effects of white disks (Figure 4B).

Background motion on the retina inhibits flight

Further information was obtained from presenting the looming stimulus on a patterned background. Certain RGCs are strongly suppressed by image motion in the receptive field surround, whereas other types of RGC are unaffected (Zhang et al., 2012; Olveczky et al., 2003; Roska and Werblin, 2003). To evoke this condition we surrounded the expanding disc with a stripe or checkerboard pattern. As the eye jitters

during the locomotion of the animal this induces large-scale image motion on the retina; for good measure we also added a steady drift to the background pattern. These global motion stimuli significantly reduced the occurrence of sub-second flight events, increased the flight latencies (Figure 4F, K), and increased the frequency of exploratory rearing events (Figure 4H). This result favors the involvement of so-called “object motion sensitive” cells that are inhibited by global motion on the retina (Zhang et al., 2012; Olveczky et al., 2003).

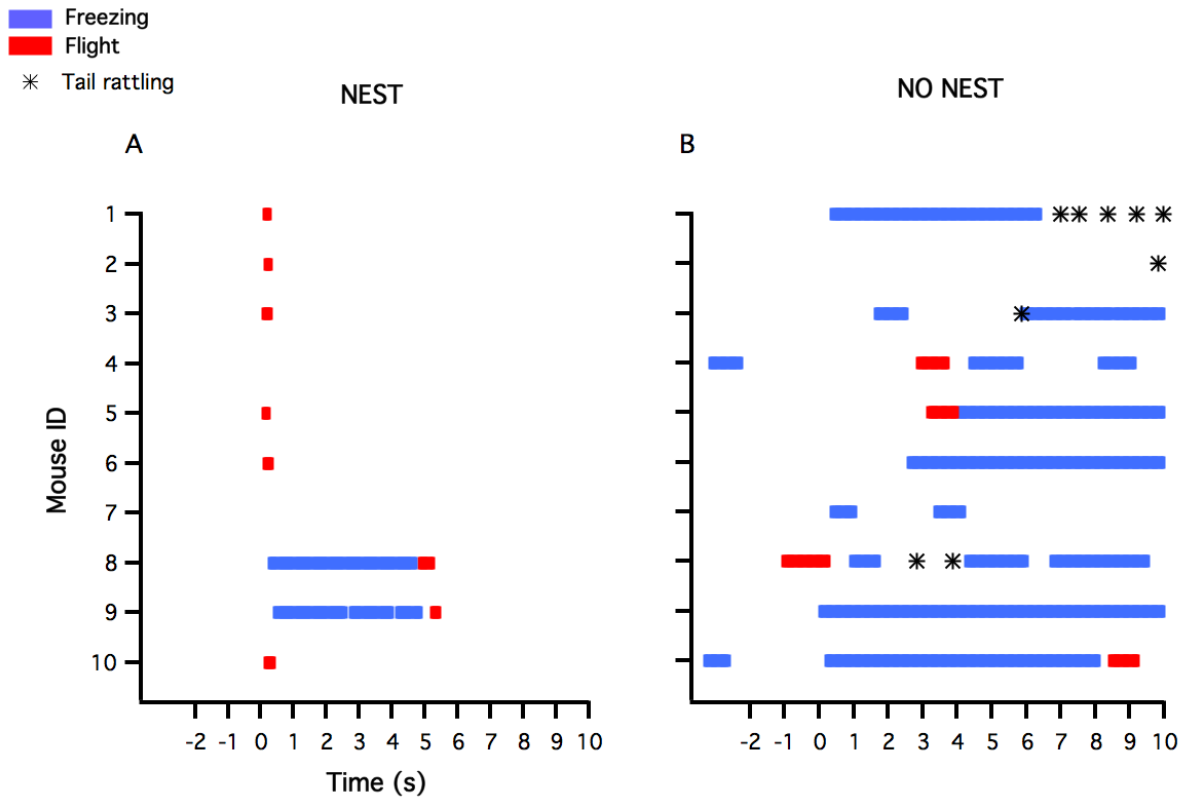
The presence of a hiding place influences the response to the looming stimulus

Animals choose to either flee or freeze in response to the looming object. Previous work on rats and mice has shown that availability of an escape a major determinant in the decision to freeze or flee in the face of a human or anesthetized conspecific threat (Blanchard et al., 1986; Blanchard and Blanchard, 1989; Blanchard et al., 1998). We asked whether presence of the nest influences the decision to flee of freeze in response to a looming stimulus. In order to test this we showed the looming stimulus to ten animals with and without the nest. When these animals were first tested with the nest 6 out of 10 showed rapid flight and 2 showed freezing followed by flight (Figure 5A). On a separate day the nest was removed and the animals were shown the looming stimulus again. This time nine out of ten animals chose to freeze. In these experiments flight was defined as a rapid run to any four corners of the arena. Two of these nine animals ran to a corner before freezing (Figure 5B, animal 4 and 5) and one fled after a long freeze (Figure 5B, animal 10). To make sure that the freezing was not due to the repeated testing, we tested some animals without the nest only and these animals also preferred freezing over flight (data not shown). Another interesting behavior emerged when the

nest was removed from the arena: 4/10 animals shook and hit their tail rapidly against the floor: a behavior known as tail rattling (Figure 5). None of the animals did this when the nest was present. Tail rattling is commonly observed during agonistic interactions and is considered an aggressive posture (Hammamieh et al., 2012; Bigi et al., 1994; Lumley et al., 2004). In our assay animals could be displaying a “fight” response in the absence of a “flight” route. Tail rattling could also be used as an “alarm call” in the face of a threat because it makes a loud noise.

This experiment shows that the flight vs freezing decision is influenced by external factors such as the presence of a nest. Ecologically this makes sense since freezing is a better strategy to avoid detection by a predator than moving around when there is no hiding place. Future experiments aim to investigate this further by systematically varying the distance of the nest to the animal at the time of the stimulus presentation. These experiments were done by Margaret Lee, an undergraduate summer student whom I mentored. The analysis were done by myself and Margaret.

Figure 5: The decision to freeze or flee depends on the presence of a nest in the arena



(A) Flight (red) and freezing (purple) and tail rattling (*) reactions of ten animals tested with a nest in the arena. (B) Reactions of same animals in (A) in the absence of a nest. Stimulus comes on at time = 0 and repeats for 10 times as shown in Figure 1.

Animals show consistent responses over days, but habituate to the presentation of continuous looming over minutes

Habituation is the decrease in the response that occurs when an initially novel stimulus is presented repetitively. We asked whether the initial looming reactions habituate with repetitions of the stimulus. We tried four regimes of stimulus presentation:

1. 10 repetitions of the looming stimulus (1 per second, the same as all previous experiments) presented each day for 5 consecutive days (n = 4)
2. 10 repetitions of the looming stimulus repeated 5 times with 1 hour intervals (n =2)
3. 10 repetitions of the looming stimulus repeated 5 times with ~ 5 minute intervals. In this scenario the stimulus was triggered as soon as the animal came to the middle of the arena after leaving the nest. Therefore the gap between repetitions varied but none exceeded 7 minutes (n=2).
4. 360 repetitions of the looming stimulus presented continuously for 360 seconds (In this condition there is no nest in the arena because this experiment was originally designed for a separate purpose.) (n = 3)

Animals showed highly reliable responses across days with no sign of habituation (Figure 6 A- E). One of the two animals stopped responding after the third stimulation when the stimulus was presented with 1 hr intervals (Figure 6 F-J, mouse 30). When stimulated within the same session with ~ 5 minute intervals the two animals tested showed highly reliable responses across trials (Figure 6 K-O). Altogether these results suggest that 10 repetitions with 1 second interval is not effective to induce any kind of

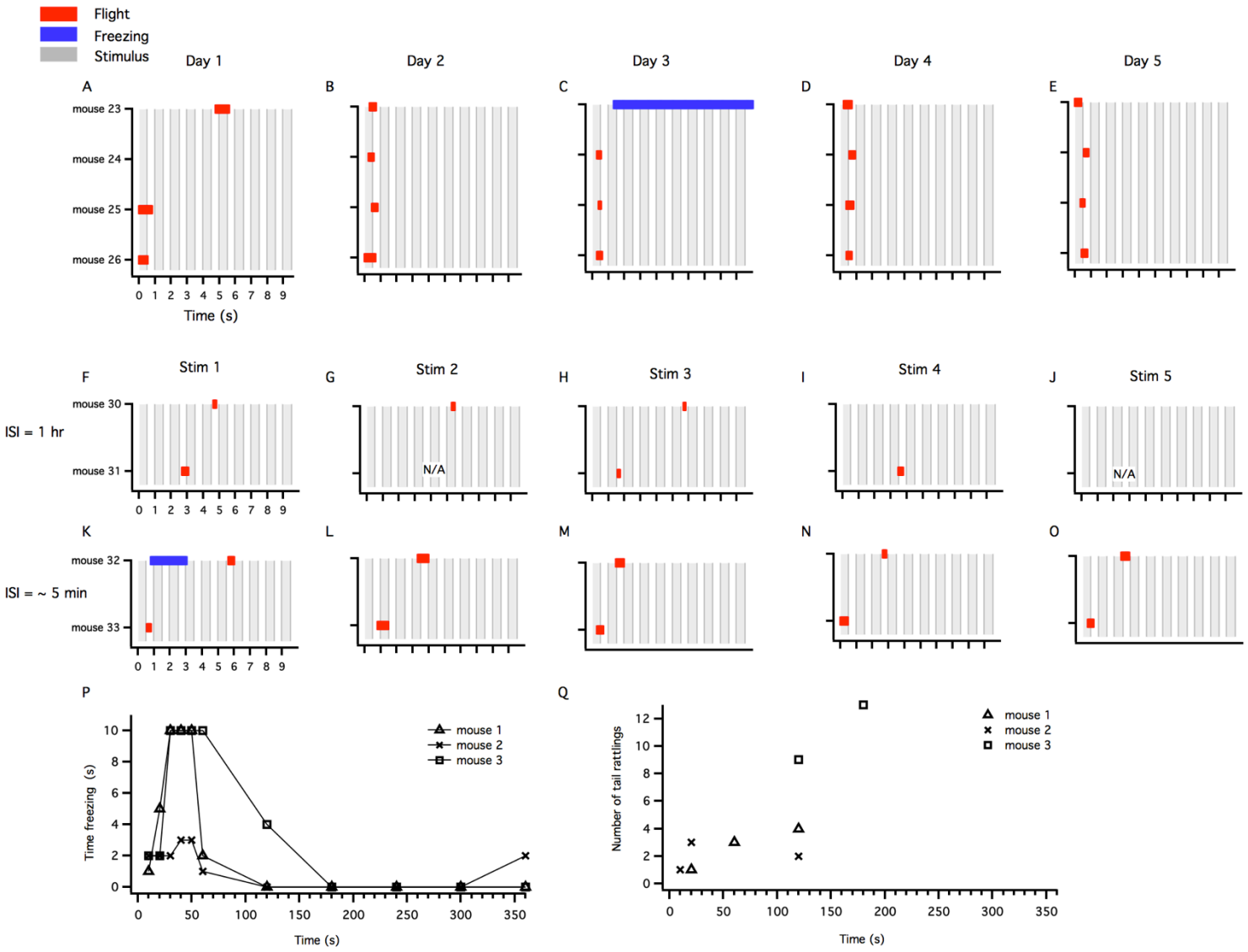
plasticity when repeated over days, hours or minutes. By contrast when animals were exposed to the stimulus regime #4, where they received continuous stimulation for 6 minutes, the responses habituated rapidly (Figure 6 P). These experiments were done with another intention and thus there was no nest in the arena, which biased the responses towards freezing. After the 120 seconds animals did not show any extended freezing or tail rattling, a behavior often observed in the absence of a nest in response to the looming stimulus (Figure 6 Q). These results suggest that continuous stimulation with 1 second intervals for at least 2 minutes is necessary to induce habituation of the freezing response.

These results are significant from two perspectives. From a practical point of view, the highly reliable responses over long periods allow one to test animals multiple times and obtain more data from single animals. From a scientific perspective, they pose an interesting question as to how the nervous system integrates information over different time scales.

Figure 6: Looming responses habituate with repetitive stimulation only with short inter-stimulus intervals

(A-E) Looming responses of four mice (23-26) on 5 consecutive days. (F-J) Responses of 2 mice to stimulus repeated every hour for 5 hours. (K-O) Responses of two mice when stimulus was repeated with intervals close to 5 minutes. (P) Histograms of three mice, showing time spent freezing in 10 second bins. Stimulus was repeated every second for 360 seconds. (Q) Histogram of tail rattling episodes in the same three mice as in (P).

Figure 6 (continued): Looming responses habituate with repetitive stimulation only with short inter-stimulus intervals



Using c-fos as a marker for neural activity in the superior colliculus

As a first step to understand which brain regions are involved in the looming responses, we looked at the expression of c-fos after exposure to the looming stimulus. C-fos is a proto-oncogene that gets activated after neural activity. The protein product can be detected with histological techniques (Bullitt, 1990). Almost all RGCs project to the superior colliculus (SC) (Vaney et al., 1981). In addition this region has been implicated in mediating approach and avoidance behaviors (Dean et al., 1989; Westby et al., 1990; Ellard and Goodale, 1988). Therefore we asked whether c-fos is expressed in SC after exposure to the looming stimulus. The goal of this approach was to identify regions of the brain that show activity and use this information for more directed circuit studies in the future.

Since there was no previous protocol for detecting a c-fos signal in the context of the looming assay, we spent some time optimizing the parameters. Table 1 summarizes the three different experimental conditions we tried. For all experiments we used a set-up with no nest for simplicity and thus biased the behavior towards freezing. Though some c-fos signal was observed in the SC in all experiments, this was not convincingly higher than the signal observed in the control animals, which were not exposed to the looming stimulus. Therefore these experiments are inconclusive at this stage. In this section I will present some preliminary data obtained from different protocols and discuss possible improvements to the method.

Table 1: Experimental conditions for c-fos experiments (Figure 7)

Experiment No	Acclimation	Stimulus	Behavior
1	None	360 x 1 Hz (360 second total)	Brief freezing followed by flight to corner and extended freezing + tail rattling
2	30 minute dark adaptation	360 x 1 Hz (360 second total)	Tail rattling+ freezing
3	3 day, 15 min habituation to the arena before stimulus	360 x 1 Hz (360 second total)	Long freezing, tail rattling

In order to increase the chance of detecting a c-fos signal we first increased the number of stimulus presentation from 1 x 10 (Figure 2) to 5 x 10 repetitions. With this condition no excess c-fos signal was detected in the animal compared to a control animal that had not been exposed to a stimulus (data not shown). We then increased the exposure time to 360 seconds where the stimulus was repeated every second (Experiment 1). Under this condition some elevated c-fos signal was observed in the medial superficial layers as well as the intermediate layers and deep layers (Figure 7 B). Unfortunately, no control tissue was available in this case for a proper comparative analysis. To reduce background signal due to visual input other than the looming stimulus we tried dark adapting mice for 30 minutes before presenting the looming stimulus and used the same experiment regime. Under these conditions (Experiment 2), c-fos expression was slightly more localized to the medial intermediate and deep layers of the SC in the experimental mouse compared to the control mouse that wasn't exposed to the stimulus (Figure 7 C,D). However there was also substantial baseline c-fos signal in the control

mouse. In addition no obvious signal was detected in the visual superficial layers. In a separate experiment, (Experiment 3), we acclimated the animals to the arena for 15 minutes without visual stimulation for 3 days prior to stimulus presentation. The acclimation regime is intended to reduce the background activity produced by being in a novel arena. The three day habituation protocol did not improve the signal to noise ratio and the results were unsatisfactory (data not shown). Behavioral reactions of these three animals to the looming stimulus are shown in Figure 6 P and Q, where experiment number match the mouse numbers (Experiment 1 = Mouse 1). In summary they all showed freezing and tail rattling reactions in the first two minutes of the stimulus presentations. Mouse 3 had the strongest responses.

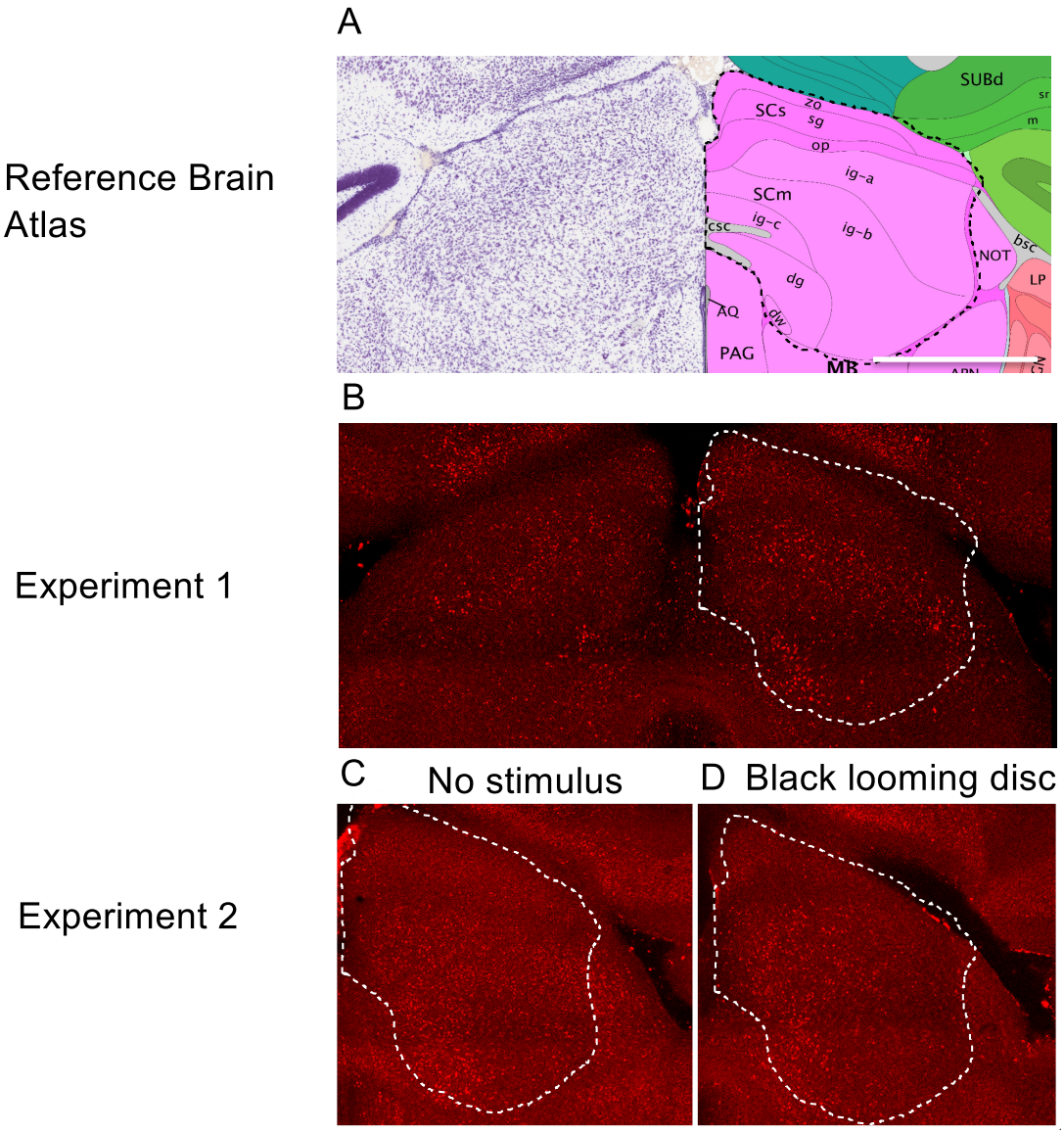


Figure 7: Preliminary c-fos staining in the superior colliculus

(A) Reference coronal section from Allen Brain Atlas with superior colliculus. (http://atlas.brain-map.org/). Superior colliculus is highlighted with dashed lines. SCs: Superior Colliculus superficial, zo: zonal layer, sg: superficial gray, op: optic layer. SCm: motor related intermediate gray layer, ig: intermediate gray layer, dg: deep gray layer. anti c-fos staining after (B) Experiment 1, (C-D) Experiment 2. Experiment descriptions are in Table 1. Scale bar: 1197 micron.

Although we detected a c-fos signal in the superior colliculus, we did not see a consistent and obvious difference in expression between control and experimental animals. There could be several reasons for this: (1) We did not control for the motor activity or visual input of the animal prior to the presentation of the looming stimulus. Superior colliculus could be active when the animal is exploring a novel arena and is certainly getting a lot of visual input. This makes it hard to differentiate any signal due to the brief looming reaction from the baseline activity of the colliculus with c-fos. We tried putting the animal in a small dark box for a few hours prior to the experiment to restrict both its movement and visual experience. However this treatment unexpectedly weakened the looming reactions. Therefore we did not pursue this strategy further. Perhaps a temporally precise detection method will be more useful. (2) The c-fos method is very sensitive to staining parameters. Some of our experiments were not interpretable simply because the control sections or images were qualitatively different from the experimental sections though care was taken to process everything equally.

Future studies should aim to use an activity marker that is more temporally precise and/or has a higher signal to noise ratio. One possibility is the catFISH method (cellular compartment analysis of temporal activity by fluorescent *in situ* (Guzowski et al., 1999)). This method utilizes the unique transport dynamics of the immediate early gene *Arc* mRNA to detect activity changes in neurons with a resolution of 30 minutes. Nuclear localization of *Arc* mRNA indicates activity within 5 minutes whereas cytoplasmic *Arc* mRNA indicates activity within 30 minutes. Differential localization of *Arc* could distinguish signals due to exploration of a novel environment and signals due to the

looming stimulus presentation. One could also try using transgenic mice where c-fos or other immediate early genes have been linked to a reporter gene such as LacZ (Smeyne et al., 1992) or synthetic activity dependent promoters (Kawashima et al., 2013) to improve signal to noise ratio.

Medial superficial layers as well as the intermediate and deep layers of the SC have been shown to evoke defensive motor behaviors in response to both electrical and chemical stimulation (Sahibzada et al., 1986; Dean et al., 1988). In addition neurons in the superficial layers have been shown to respond robustly to the looming stimulus (Zhao et al., 2014). If signal to noise ratio can be improved with suggested techniques above, new experiments can guide future studies for more specific cell targeting and electrophysiology.

All work in this section was done by Zeynep Turan, a rotation student who worked with me.

Discussion

In conclusion, we found that mice execute robust flight and freezing behavior in response to the visual display of an approaching object. We showed that the probability of flight and rearing behaviors depends strongly on the parameters of the visual stimulus, suggesting that specialized visual channels are involved in transmitting the relevant information. The results are significant from multiple perspectives.

Firstly, they present a novel addition to the repertoire of visually guided behaviors in the laboratory mouse. This animal model is increasingly popular in visual neuroscience, including basic studies of processing at all levels of the visual system, and translational research on neural regeneration and recovery. However, the efforts to relate neural circuits to behavior are currently hampered by the scarcity of behavioral assays of visual function. Among the ~20 types of RGCs in the mouse retina some are known to support very specific behaviors. For example, melanopsin cells regulate circadian entrainment and the pupil constriction reflex (Chen et al., 2011), whereas ON-DS cells project specifically to nuclei of the accessory optic system, responsible for the optokinetic reflex (Yonehara et al., 2009). The looming response described here can also be viewed as an essential reflex, presumably for avoiding aerial predators. It occurs on the animal's very first exposure to the stimulus, and sports the same reliability and sub-second reaction time as the pupil and optokinetic reflex. This suggests that the looming response, too, may originate in a dedicated retinal module.

Second, our experiments suggest which may be the relevant retinal circuits. The comparison of dark and bright discs clearly points to a role for the OFF ganglion cells. Among these one distinguishes two prominent types with sustained or transient responses (Pang et al., 2003). They both have receptive fields of ~10 deg diameter (Sun et al., 2002), so the stimulus at the optimal speed of 35 deg/s sweeps over the receptive field in ~0.3 s and the ineffective slower stimulus in 3 s (Figure 4J). A sustained cell will fire under both conditions, whereas a transient cell prefers rapid changes, and will thus fire more weakly to the slow stimulus. Thus the observed speed-dependence of the looming response favors a transient OFF channel, such as the PV-5 neuron (Munch et al., 2009) whose firing is indeed strongly driven by dark expanding objects. Other candidate pathways include ganglion cells with ON-OFF responses, such as the ON-OFF direction-selective cell (Weng et al., 2005), or the W3 cell (Zhang et al., 2012). The latter has a transient response, stronger for OFF than for ON events; is concentrated in the inferior retina, which monitors events above the animal; and is strongly suppressed by surround motion, much like the looming response itself. All these candidates project to the superior colliculus, a central station that mediates approach and avoidance behaviors (Sahibzada et al., 1986). Of course it is possible that the stimulus-specificity instead arises from more central visual processing. Future experiments with genetically modified mice can test the involvement of specific neural circuits in this behavior.

Third, from a methods perspective, the behavior described here presents an interesting handle for the study of innate defensive responses. Predator avoidance in mice has

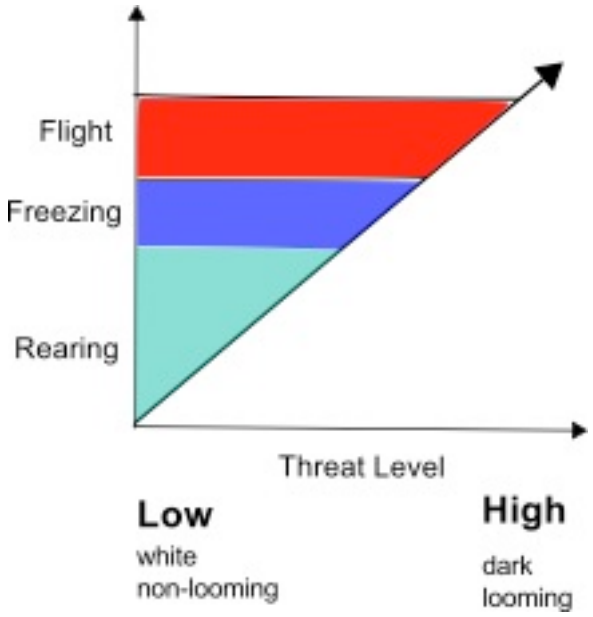
been studied extensively using real predators (Blanchard et al., 1998) and their odors (Apfelbach et al., 2005; Sotnikov et al., 2011). By contrast the looming assay relies exclusively on visual cues and offers much greater control of the stimulus parameters. As documented here, one can systematically vary luminance, contrast, speed, and other features of the stimulus, and obtain a modulation of the defensive behavior. Taking advantage of accurate temporal control of the stimulus we observed innate flight responses with latencies as low as 250 ms, shorter than reported for noxious foot shock (Muller et al., 2011) or even for exposure of wild mice to real rats (Blanchard et al., 1998). This short reaction time for the entire sensory-motor loop sets limits on the amounts of central processing involved in the behavior.

Our survey of different visual stimuli and the behaviors they trigger provides insight in the potential neural mechanisms that could be responsible for translating the visual input to behavioral output. The black looming stimulus was the most effective in driving defensive responses and inhibiting the exploratory rearing behavior. On the other hand the white receding disc was the least effective in triggering defensive behaviors and triggered the rearing behavior the most effectively despite the fact that this stimulus consists of dark moving edges. This suggests that the key features of the stimulus for biasing the behavior towards defensive behaviors and inhibiting exploratory postures are the dark contrast and the expanding motion. The dark looming disc contains both of these features, and thus triggers the strongest responses; white receding disc contains none of these features and thus predominantly elicits non-defensive behaviors. White looming and dark dimming discs trigger intermediate responses of rearing with

occasional flight or freezing. It would be interesting to look for neurons in the visual pathway that encode specifically these features of the stimulus that are behaviorally relevant. One example is a neuron that responds to looming regardless of the polarity of its contrast (white and dark looming disc). Contrast invariant neurons encoding specific visual features are identified in many places of the visual system (Anderson et al., 2000; Rolls and Baylis, 1986).

How do these features give rise to the behavior that is either exploratory or defensive? One possibility is that stimulus features with different threat values are integrated to calculate an overall threat value for the animal, which would determine the choice of action. Stimuli with low threat value such as white or non-looming objects trigger exploratory postures; however if the threat value exceeds a threshold, defensive postures are triggered (Figure 8). “Predator imminence” theory proposes such a transition from freezing to flight as the predator imminence increases (Blanchard et al., 1998). Scalability of behavior has also been shown in the context of aggressive behaviors in responses to increased optogenetic stimulation of the ESR1+ hypothalamic neurons (Lee et al., 2014). The evidence for scalability is present in our data, where there are plenty of instances where the exploratory rearing is transformed into freezing or flight as the stimulus persists (Figure 4). Though flight reactions did not require prior freezing, freezing reactions were almost always followed by flight to nest.

Figure 8: Threat level is determined by the features of the visual stimulus and determines the behavioral outcome



Though it was not the focus of this study, we were intrigued by the observation of extended freezing in response to these visual displays (Fig. 3A; Supplementary Movie 2). Freezing in laboratory mice has been reported in response to aversive ultrasonic cues but only in highly stressed animals (Mongeau et al., 2003), or more commonly as a result of context conditioning after repetitive foot shocks (Wiltgen et al., 2001). Unconditioned exposure to a predator odor does not elicit extended freezing (Sotnikov et al., 2011; Apfelbach et al., 2005). Even exposure to real rats produces only temporary immobility interspersed with active movements (Griebel et al., 1996) One may speculate that freezing is uniquely effective as a defense from aerial predators, which can detect the mouse only by vision or audition. Freezing eliminates both the visual motion that distinguishes the target from the background, and the rustling that might give it away acoustically. By contrast, once a predator is close within olfactory range, escape may be the more effective strategy (Fanselow, 1994).

The defensive responses of mice to the looming stimulus are remarkably robust with close to 100% probability of a response at first exposure. However we show that these responses are also flexible depending on stimulus history (Figure 6). We show that the strength of habituation varies with inter-stimulus interval. 6 minutes of 1 Hz stimulus exposure causes habituation of the response while longer inter-stimulus interval or lower number of repetitions does not. In this respect the looming avoidance in mice is reminiscent of defensive reflexes of lower order vertebrates and invertebrates (Hinde, 1954; Clark, 1960; Pinsker et al., 1970). Both the famous gill withdrawal reflex of

Aplysia and withdrawal reflex of the tube worm *Nereis* habituate with repetitive stimulation. The strength of the habituation is dependent on the inter-stimulus interval in both cases (Clark, 1960; Pinsker et al., 1970).

Another interesting aspect of habituation is the effect of stimulus strength on strength of habituation. Greater habituation is observed with weaker stimuli in both *Aplysia* and tube worm *Nereis* (Clark, 1960; Pinsker et al., 1970). Interestingly in the latter, responses to moving dark shadows habituate slower than responses to a mechanical shock (Clark, 1960) arguing that dark shadow is a more effective stimulus. In our experiments such a phenomenon was apparent when white looming disc and black looming disc were presented consecutively on multiple days to the animals. We observed that the animals habituated to the weak stimulus, white looming disc, rapidly the second day even when they still showed robust responses to the black looming disc within the same experiment session (data not shown).

Thompson and Spencer described nine characteristics of habituation based on the hindlimb reflex of the acute spinal cat (Thompson and Spencer, 1966). The looming responses of mice exhibit three of these properties: (1) Responses decline exponentially as a function of stimulus repetitions, (2) Habituation is stronger with shorter inter-stimulus interval and (3) weaker stimulus strength. Another property they stress is the generalization of habituation to other stimuli. Based on our experiments with white and black looming discs, this does not hold for the looming response. But experiments with different types of stimuli would better illuminate the point. It would be

interesting to ask whether the other five parameters apply to the looming response. Shortly these are: (1) restoration of the habituated response with rest, (2) potentiation of habituation with series of habituation and recovery, (3) prolonged habituation training resulting in a delayed recovery, (4) dishabituation of the response upon presentation of another strong stimulus, (5) habituation of dishabituation upon repeated of dishabituation trainings. Similarities in the properties of habituation observed across species suggest common neural mechanism. Comparisons of neural mechanisms across species could be a fruitful place to start to investigate the site of habituation in the mouse brain.

It is of great interest to understand the neural circuitry that starts from the retina all the way to the muscles to produce the reliable defensive responses to the looming stimulus. The next chapter will explain our efforts to identify specific cell types in the retina that are necessary for triggering defensive behaviors.

Experimental Procedures

Looming Behavior

The behavioral arena was an open-top plexiglass box, 48 cm wide x 48 cm deep x 30 cm high. The floor and three walls were covered with a matte spray-on coating (Krylon) to prevent reflections of the stimulus. The nest was in the shape of a triangular prism 20 cm wide x 12 cm high. The arena had dim lighting from the gray screen of the monitor. Four bright LEDs (Marubeni America Corporation, L810N-66-60) provided infrared illumination for video recording that was invisible to the mouse. Two cameras, one from the top, one from the side (PointGrey, Flea3 firewire, monochrome) were triggered simultaneously to record the mouse's movements at 30 fps. The stimulus was programmed in C++ using OpenGL libraries and was displayed on a LCD monitor (HPZR22w).

Mice of strain C57BL6/J from Jackson Labs aged 8-12 weeks were group-housed and maintained on a 13 h light / 11 h dark cycle. No significant difference in frequency or latency of flights was observed between males and females or between animals tested during day and night. Following these pilot tests, all experiments were performed on males and during the day, when the animals generally seemed less anxious.

The stimulus was triggered by the experimenter when the animal entered a black square in the middle of the arena. When multiple conditions were compared on the same animal (Figure 4), we conservatively presented the looming stimulus last. Thus any form of habituation would have its strongest effect on this reference condition. Each animal was used only once per experimental condition, and results are reported from a

total of 40 animals: 10 for looming stimulus only (Figure 4), 10 for comparison with the 3 disc display conditions, 10 for testing effects of background motion and 10 for testing the effect of different speeds (Figure 4).

The video recordings were analyzed with a custom-written Matlab program that uses background subtraction to locate the mouse. The velocity was calculated and smoothed with a median filter. Freezing is defined as the episodes of 2 s or more where the velocity is less than 15% of its average value over the 10 second interval prior to the stimulus onset. Flight is defined as episodes where the velocity is greater than 4 times the average and the animal's final position is in the nest. Upright rearing events were scored by hand as episodes when the mouse raises both front paws and puts them back on the ground.

The ethograms (Figure 3A, 4A-D) mark episodes of flight, freezing or rearing as a function of time before or after stimulus onset. Statistical significance (Figure 3D, 4E-K) is labeled as: * $p<0.05$, ** $p<0.01$, *** $p< 0.005$. These p-values were derived from a z-test for the equality of two proportions for flight and freezing (binomial distribution); z-test for the equality of two counts for upward rearing (Poisson distribution); one-sided sign test for latency comparisons. For animals that did not flee within 10 s of stimulus onset, a flight latency of 10 s was assigned.

Thanks to a move of the laboratory over the course of the study we could compare the behavior of mice housed in two different animal facilities: at Harvard and Caltech. Both

sets of animals exhibited fast and robust defensive reactions to the looming disc, but with some significant differences. The Harvard animals reacted with escape every time, with no occurrence of freezing, unlike the animals housed at Caltech. Furthermore, the Harvard animals showed much weaker reactions still to the alternative disc displays (Figure 4B-D). For a conservative and internally consistent report we only show data from the Caltech animals. We suspect that differences in the internal state of the animals owing to housing conditions in the two colonies underlie the different outcomes. This points to a need for standardization in animal care if one wants to compare behavioral results across laboratories.

c-fos staining

Behavioral experiments were conducted as described in Table 1. After each experiment the animal was placed in an empty cage for 1.5 hrs, after which it was perfused with 4% paraformaldehyde (PFA) under ketamine and xylazine anesthesia. Brains were extracted and fixed overnight in 4 % PFA at 4C. For cryostat sectioning, brains were then placed in 30% sucrose in PBS and kept at 4^{o}C. 50-100 µm sections were collected in PBS- 2.5 to 5 mm from Bregma. Floating sections were stained with goat anti c-fos (Santa Cruz, 1:1000) for 24 hours, followed by Alexa 647, or 488 secondary antibodies (Life Technologies, 1:1000). Sections were mounted on glass slides using Vectashield (Vector Labs) mounting medium and were imaged with LSM 710 Confocal microscope under 10X magnification.

Chapter 2: Mapping retinal channels to innate visual behaviors of mice

Summary

Retinal ganglion cells constitute parallel information channels that process the image and send this information to the brain. Despite extensive physiological and anatomical knowledge of these different cell types, how each channel contributes to different behaviors is less understood. Here we investigate the behavioral roles of two different retinal channels: alpha RGCs and direction selective RGCs (dsRGCs) using cell-type specific ablations. We test the effects of the ablations on three innate behaviors: looming avoidance response, optokinetic reflex (OKR) and pupillary light reflex (PLR). The causal connection between the direction selective cells and OKR has been previously established (Yoshida et al., 2001), and thus serves as our positive control. We show that the ablation of direction selectivity impairs the OKR and leaves the looming response and pupil response unaffected. By contrast ablation of alpha cells significantly reduced looming avoidance behaviors, had a mild effect on OKR and caused a delay of the pupil response. Interpretation of these results is complicated by a recent discovery that our method of ablation affected a larger population of cells than just alpha cells. We are currently working to understand the cause of this larger cell loss and methods to target alpha cells more specifically. These efforts are detailed in the discussion section.

Introduction

There are ~20 types of RGCs in the mammalian retina, each of which extracts a specific feature of the visual scene (Masland, 2012). Ablation studies have revealed differential roles of some RGC types mostly in behaviors that depend purely on light intensity. For instance a subtype of the M1 intrinsically photosensitive RGCs (ipRGCs) provides the main input to the olivary pretectal nucleus (OPN) and drive the pupillary light reflex (PLR) while another subtype projects to suprachiasmatic nucleus (SCN) and regulates circadian rhythms (Chen et al., 2011). Similarly the ON type direction selective cell projects specifically to the terminal nuclei of the accessory optic system (AOS), which is responsible for the optokinetic reflex (Yonehara et al., 2009). What about other RGC types that make up approximately 90% of all RGCs and project to areas relevant to pattern vision like the superior colliculus (SC) and lateral geniculate nucleus (LGN)? Almost nothing is known about their behavioral roles due to scarcity of innate visual behavioral assays and cell specific targeting methods. In this report we made use of two cell-specific Cre lines, Kcng4^{Cre} (Duan et al., 2014) and ChAT^{Cre} (Duan et al., 2014) to target alpha cells and direction selective cells respectively via Cre-dependent diphtheria toxin receptor mediated ablations ((Hatori et al., 2008), Experimental Methods). The roles of two retinal channels are investigated in three innate visual behaviors: looming response, optokinetic reflex and pupillary light reflex.

Direction selective retinal ganglion cells (DSRGCs) respond preferentially to a specific direction of motion, commonly referred to as the “preferred” direction, while responding little to the opposite of that direction, the “null” direction. These cells make up ~ 20% of

all RGCs (Kay et al., 2011; Bleckert et al., 2014; Yonehara et al., 2009). They have three types based on their light responses and lamination patterns of the dendrites: ON, OFF and ON-OFF cells. ON-OFF DS cells were first described in the rabbit retina as they differed from the classically concentric cells in two aspects (1) they responded to both onset and offset of light and (2) they responded to motion only in a specific direction (Barlow and Hill, 1963; Barlow et al., 1964; Vaney et al., 2012). These cells have four subtypes that respond to anterior, posterior, inferior and superior motion respectively (Oyster and Barlow, 1967). Later this type was described in the mouse (Weng et al., 2005; Kay et al., 2011). Shortly after ON-OFF cells another type of DS cell that responds only to the onset of light was identified in the rabbit retina (Oyster and Barlow, 1967; Oyster, 1968) and recently in the mouse retina (Sun et al., 2006; Yonehara et al., 2009). These so called ON DS cells are fewer in number, respond to slower speeds of motion than the ON-OFF type and have only three subtypes that respond to anterior, downward and upward motion (Oyster and Barlow, 1967; Oyster, 1968; Sun et al., 2006; Yonehara et al., 2009). The OFF DS cell has been identified in the mouse retina and has a selectivity for upward motion (Kim et al., 2008).

The mechanisms of direction selectivity has been extensively studied in the DS cells. Most studies focused on the ON-OFF type, though ON type was shown to use similar mechanisms (Sun et al., 2006; Yonehara et al., 2011). In their classic study in the rabbit retina, Barlow and Levick established the key role of inhibition in providing direction selectivity for the cell (Barlow and Levick, 1965). Later studies showed that the source of the inhibitory input to the DS cell was the starburst amacrine cell (SAC): a cholinergic

and GABAergic interneuron. SACs have their cell bodies in two different layers in the retina and their dendrites co-stratify with the dendrites of the DS cells in the inner plexiform layer of the retina. Electrophysiology, calcium imaging and structural imaging by electron microscopy all revealed that SACs are connected asymmetrically to the DS cells; providing inhibition preferentially from the null direction side. In addition SAC processes are themselves direction selective responding preferentially to centrifugal motion (Fried et al., 2002; Euler et al., 2002; Briggman et al., 2011). It has also been shown by somatic patch clamping techniques that asymmetric excitation to the DS cells contribute to direction selectivity (Fried et al., 2002; Taylor and Vaney, 2002; Borg-Graham, 2001). However this is still not well established due to the caveat of voltage-clamp errors in these studies (see Vaney et al. 2012 and Demb, 2007 for review). ON and ON-OFF DS cells, both receive inhibitory input from starburst amacrine cells. The direction selectivity of the OFF DS cell however likely arises from a starburst independent mechanism (Kim et al., 2008). Ablation of the starburst cells resulted in loss of direction selectivity as well as optokinetic eye movements (Yoshida et al., 2001; Amthor et al., 2002).

Despite extensive studies of the direction selectivity mechanism within the retina, how this feature is utilized by the downstream areas of the brain is not well understood. In fact the textbook view of direction selectivity still implicates that this feature arises in the visual cortex (Hubel and Wiesel, 1962). This view is starting to change since direction selective cells are now identified both in the retina and the lateral geniculate nucleus, a relay to the primary visual cortex (Piscopo et al., 2013). It is still not known though how

direction selectivity is used in the context of innate behaviors. ON DS cells project to the nuclei of the accessory optic system (AOS), a region known to regulate eye and head movements in response to global motion, also called the optokinetic reflex (OKR) (Simpson, 1984). In addition their speed and direction tunings match well with the neurons in the AOS, making them a major contributor to responses in this region and a likely candidate for driving the optokinetic reflex. Yonehara et al. showed neuron in the medial terminal nucleus of the AOS are active during vertical optokinetic eye movements (Yonehara et al., 2009). On the other hand the ON-OFF DS cells project their axons to several regions including the superior colliculus and lateral geniculate nucleus. A subset of ON-OFF DS cells called the BD cell also has projections to the medial terminal nucleus of the AOS and the nucleus of the optic tract (Kay et al., 2011). The behavioral roles of these connections are a complete mystery.

Here we ask if direction selectivity as computed in the retina is necessary for the detection of a looming stimulus, a skill reliant on motion detection (Yilmaz and Meister, 2013). To perturb the circuitry of DS cells we selectively ablated starburst amacrine cells as labeled by the $ChAT^{Cre}$ line. Since the roles of these cells in the OKR and PLR were previously shown, (Yoshida et al., 2001) the effects of the starburst ablation on the these two behaviors serve as positive and negative controls for our experiments.

The second class of cells we investigated were the alpha retinal ganglion cells. These were initially described in the cat retina based on their large cell bodies and dendritic fields (Boycott and Wassle). Due to their neurofilament rich nature, they can be easily

identified with classical reduced silver methods or histological neurofilament markers (Peichl et al., 1987; Sun et al., 2002). Physiologically they were described as the Y (brisk-transient) cells (Enroth-Cugell, 1966} based on their non-linear receptive field properties and high conduction velocity of their axons (Cleland et al., 1971; Cleland et al., 1973). Soon after it was established that the Y cells were the physiological counterparts of the alpha cells (Cleland 1975; Fukuda 1984; Peichl, 1991).

In the mouse retina, four types of alpha cells have been identified based on different physiological properties and stratification patterns in the inner retina: ON sustained, ON transient (in preparation), OFF sustained and OFF transient (Pang et al., 2003; van Wyk et al., 2009). Their differ from the classical linear center-surround cells by the complex structure of their receptive fields. Just like the direction selective cells, their receptive fields are made up of nonlinear subunits, which makes them very sensitive to motion. They are not however direction selective (Enroth-Cugell and Freeman, 1987).

It is largely mysterious what message do the alpha cells convey to the brain. Experiments with traditional visual stimuli such as moving bars failed to identify any feature of these cell besides motion sensitivity. Recordings from the OFF-transient alpha cell, also called the PV-5 cell revealed that this cell prefers expanding motion to lateral motion making it a putative “looming detector” for the mouse (Munch et al., 2009).

One approach to understanding the message the alpha cell conveys to the brain is understanding the behavioral roles of this class. Schmidt et al. argued that the ON type alpha cell has a role in contrast detection in the context of OKR (Schmidt et al., 2014), though the authors didn't address how this could come about without a direct projection of the alpha cell to the AOS. The ON type alpha cell has projections to the olivary pretectal nucleus, however whether they have any role in the pupil reflex is unknown (Ecker et al., 2010). To target alpha cells we made use of the $Kcng4^{Cre}$ line of mice (Duan et al., 2014), which labels these cells in addition to the type 5 bipolar cells. We ask whether this is necessary for the looming avoidance behavior. In addition we shed light on the functional relevance of the projection of ON alpha cells to the olivary pretectal nucleus (Ecker et al., 2010) by inspecting the effect of the ablation on the pupil reflex.

Alpha cells and direction-selective cells diverge in their projection patterns to downstream brain areas, indicative of distinct behavioral roles (Dhande and Huberman, 2014). In this study we confirm previous findings that loss of direction selectivity results in loss of the OKR and has no effect on the pupil reflex using a diphtheria toxin mediated ablation method. We use this as a starting point and a confirmation of our ablation method to ask the following questions: 1. Are either of the motion sensitive channels, alpha cells or direction selective cells necessary for the looming avoidance behavior? 2. Are the behavioral roles of the two channels dissociable in the context of three innate behaviors? We predict that if these ganglion cell classes are dedicated to

specific behaviors, ablating them would result in defects that are specific for each type. Otherwise, we expect to observe overlapping defects for both cell types.

Results

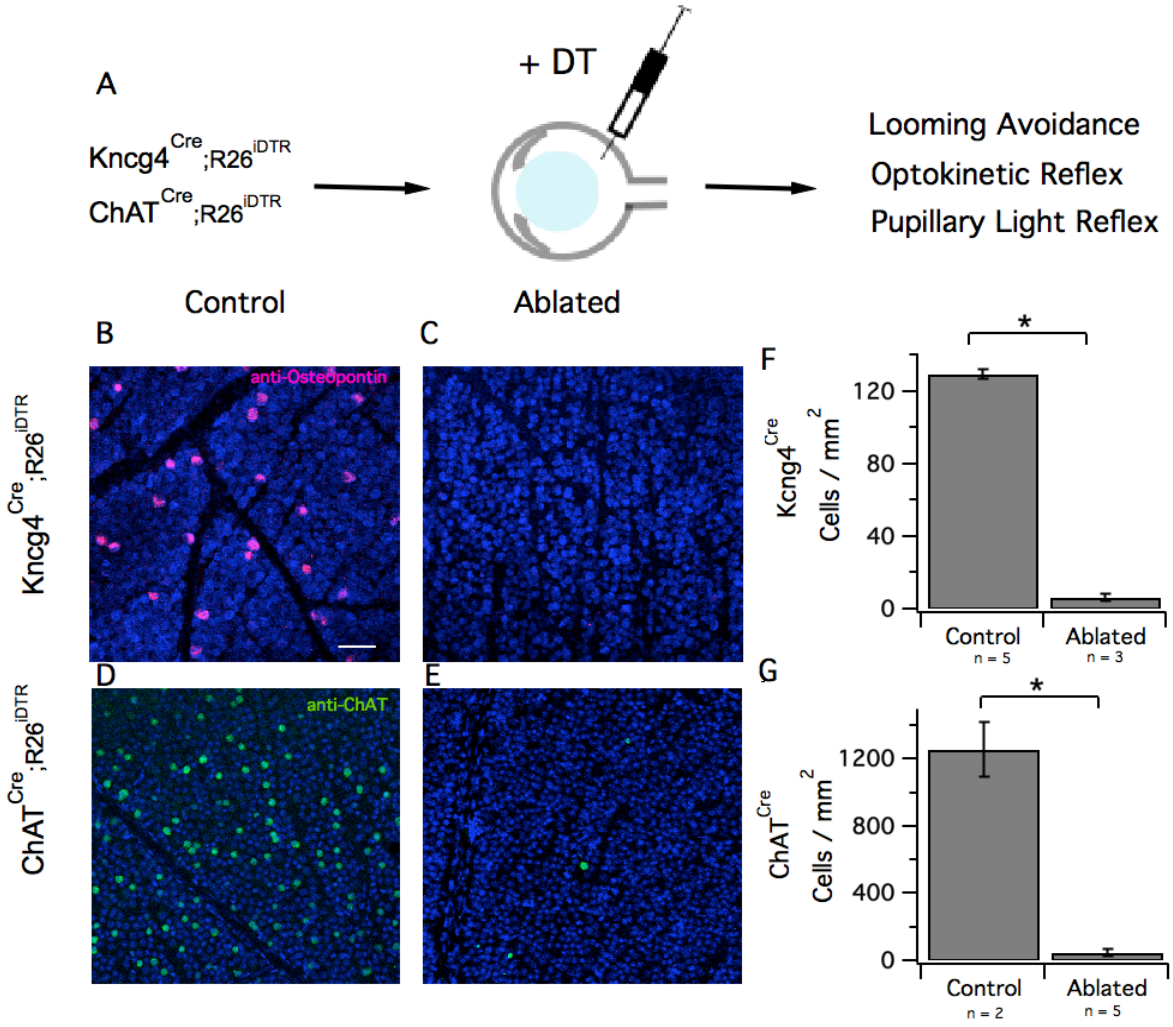
Alpha cells and starburst amacrine cells are effectively ablated using inducible diphtheria toxin receptor

To ablate alpha cells and starburst cells we crossed Kcng4^{Cre} and ChAT^{Cre} mice to a mouse that expresses diphtheria toxin receptor in a Cre dependent manner (iDTR). We injected diphtheria toxin (DT) intra-ocularly and performed behavioral testing 3-6 weeks after injections. Ablations were confirmed with cell type specific antibodies after behavioral testing was completed (Figure 9A, Experimental Procedures). There is a ~95% reduction in alpha cells after DT injections as assessed by alpha cell marker Osteopontin ((Duan, 2015), Figure 9 B,C,F). Similarly starburst cells are 90% reduced after DT injection (Figure 9 D,E,F) as determined with anti-ChAT staining.

To assess the specificity of our ablations we stained for ON-OFF direction cells and starburst amacrine cells in Kcng4^{Cre};iDTR retinas. Both of these types were intact in the ablated retinas (Figure 10). However staining vertical retinal sections revealed that type 5 bipolar cells were also ablated (Cre staining in Figure 11 C vs D) and all cellular and synaptic layers looked thinner in these retinas (Figure 11 C-F). Consistent with the whole mount results, also in vertical sections the starburst amacrine cells and their processes were intact (Figure 11 D). These results indicate that a broader population of cells than alpha cells were ablated in the Kncg4^{Cre};iDTR DT+ retinas. We are currently investigating this source of this wide cell loss. Please refer to the discussion section and Figure 18 for more details on our progress on this front. By contrast there was no

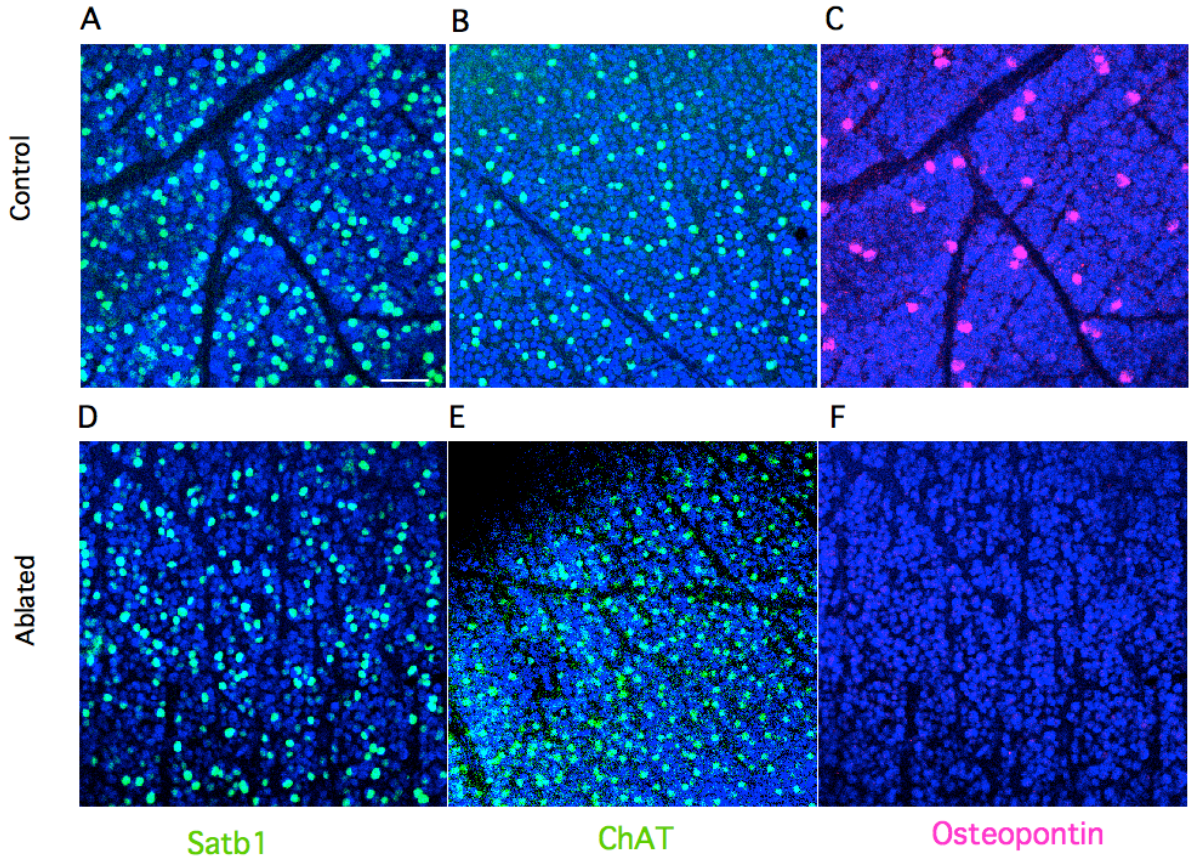
noticeable thinning in the ChAT^{Cre};iDTR retinas (Figure 11 A,B). In addition we checked for the presence of alpha cells in these retinas with the SMI-32 alpha cell marker and confirmed that these cells were intact (Figure 12).

Figure 9: Alpha cells and starburst cells are effectively ablated by diphtheria toxin



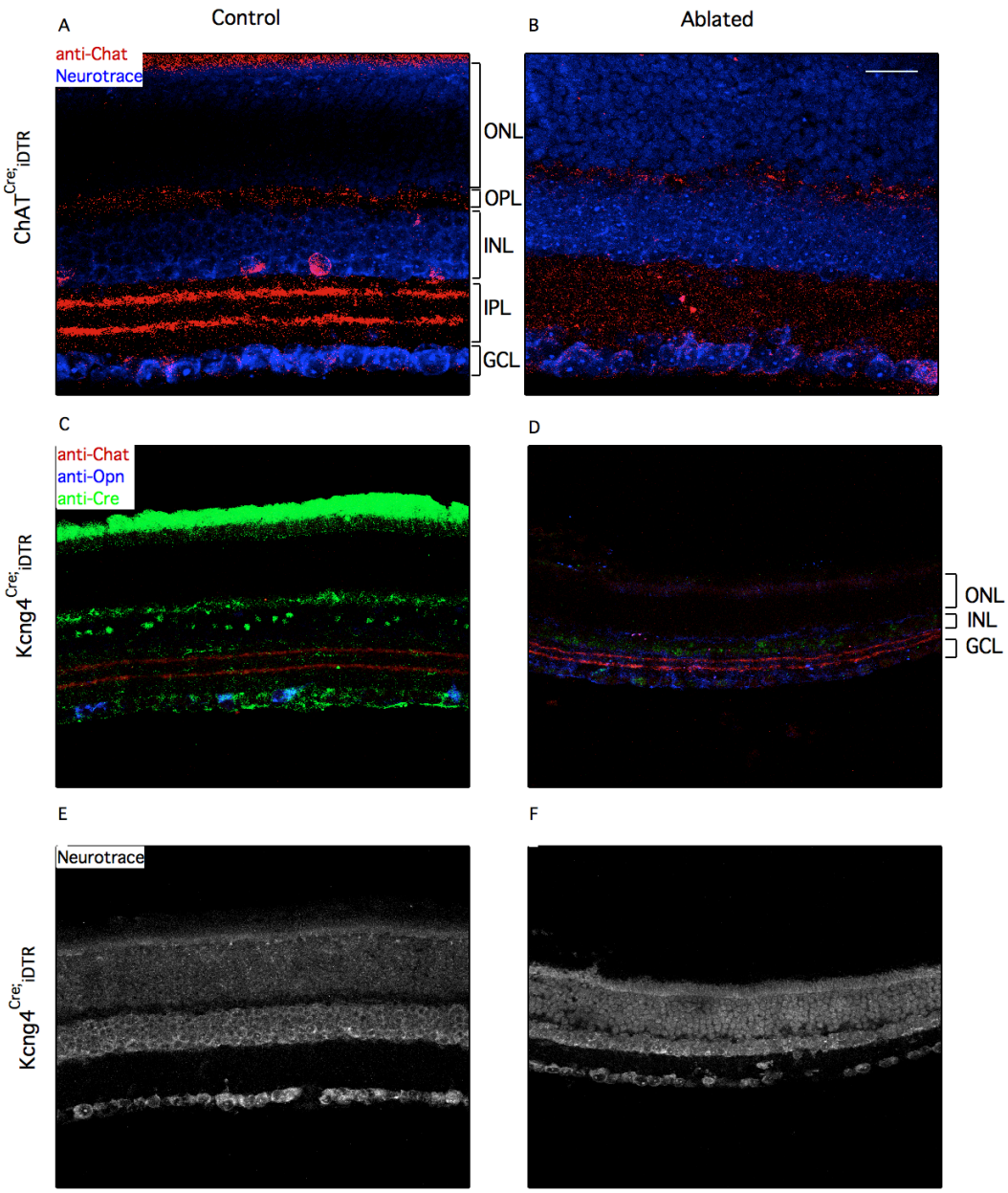
A schematic of the experimental design (A). Examples of control retinas stained with anti-Osteopontin for alpha cells (B) and anti-ChAT for starburst amacrine cells (D). Examples of ablated retinas stained with the same markers (C and E). Blue is neurotrace. Quantification of the ablation efficacy for both groups (F and G). Scale bar = 50μ. * = $p<0.5$ based on Mann-Whitney U test.

Figure 10: ON-OFF DSRGCs and starburst amacrine cells are intact in the alpha ablated retinas



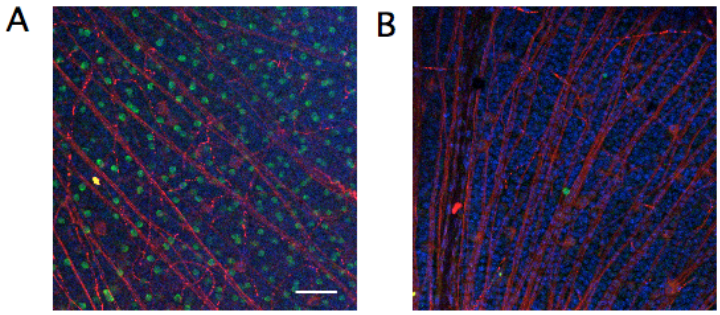
Example control retinas stained with ON-OFF dsRGC marker Satb1, starburst marker ChAT and alpha cell marker Osteopontin respectively (A, B,C). Example alpha ablated retinas stained with the same markers (D,E,F).

Figure 11: Effects of the ablations seen through retinal sections



Anti-ChAT and neurotrace staining in control (A) and ablated retinas (B) in the Chat^{Cre};iDTR line. Anti-VAChT, Anti-Osteopontin and Anti-Cre staining in control (C) and ablated retina (D) in Kcng4^{Cre}iDTR line. Nerurotrace staining in the same retinas in (E) and (F). ONL = Outer nuclear layer, OPL = Outer plexiform layer, INL = Inner nuclear layer, IPL = Inner plexiform layer, GCL = Ganglion cell layer.

Figure 12: Alpha cells and neurofilaments are intact in starburst-ablated retinas



Example control retina (A) and starburst ablated retina (B) stained with alpha cell and neurofilament marker Smi-32, starburst marker ChAT and neurotrace.

Flight and freezing reactions to a looming stimulus are significantly reduced in the alpha ablated animals but remain unaffected in starburst-ablated animals.

Animals respond to a looming dark disc in the upper visual field with either freezing or fleeing to nest (Figure 13 A, B) (Yilmaz and Meister, 2013). These reactions were significantly reduced in alpha ablated animals compared to the controls that either received a sham PBS injection or DT injection but did not carry the iDTR (Figure 13 C,D,I). In addition we tested the looming responses of six animals before and after injection. All six showed strong reactions before the ablation (Figure 13 E, F). The three that received sham injections still showed strong reactions after injection (Figure 13 G), while the three that received DT did not respond to the looming stimulus after the injection (Figure 13 H). Interestingly alpha ablated animals showed an increase in the rearing response to the looming stimulus compared to the controls (Figure 14 A,C), suggesting the animals recognized the stimulus but did not perceive it as a threat. Moreover when the same animals were shown a white looming disc, which normally evokes rearing responses (Figure 14 B), these animals showed a decrease in the rearing response compared to the controls (Figure 14 B,D). Together with the decrease in the defensive reactions in response to the black looming disc stimulus, this result suggests alpha ablated animals do not recognize overhead stimuli for both polarities as well as the controls.

All nine starburst ablated animals showed either a freezing or flight response to the looming stimulus indicating that the ablations did not affect this response (Figure 13 J, K, P). Two out of five animals that were tested before and after DT injection showed an increase of more than 1 sec in latency of the response (Figure 13 M, O), compared to zero out of five of the controls (Figure 13 L, N), but this was not statistically significant. These results indicate that cell types ablated in the Kcng4^{Cre}, possibly alpha cells, are required for robust avoidance behaviors while starburst cells are not essential.

Figure 13: Looming responses are significantly reduced in alpha ablated animals while starburst ablations do not have an effect

A schematic of the experimental set-up for the looming avoidance response (A). M = stimulus monitor, N = nest, D = distance to nest. Stimulus is the same as described in Figure 1. Flight and freezing episodes are defined as described in Figure 1 (B) (Yilmaz and Meister, 2013). Gray bars represent the repetition of the looming stimulus. Flight and freezing behaviors are demonstrated in the ethograms with purple and red bars (B). Defensive behaviors of each $Kcng4^{Cre}$ animal in the control group (C) and ablated group (D). Each row represents an animal and the grey bars represent the repetition of the stimulus in the ethograms. 6 animals have been tested before and after injection. They are indicated by * and + for control and ablated respectively. The behaviors of 3 control animals before and after injection are shown in (E) and (G). (F) and (H) show the behaviors of ablated animals before and after injection. Mean defensive behaviors (freezing or flight) are shown in (I). Flight and freezing behaviors of $ChAT^{Cre}$ ablated animals are shown in (K) and control animals are in (J). Similar to $Kcng4^{Cre}$ animals some $ChAT^{Cre}$ animals have been tested before and after DT injection and they are marked with * and + for control and ablated respectively. The before and after reactions are in (L) and (N) for control animals and (M) and (O) for ablated animals respectively. Each line represents an animal. Mean reactions for $ChAT^{Cre}$ animals are shown in (P). *** = $p < 0.005$ based on chisquare test for equality of two proportions.

Figure 13 (continued): Looming responses are significantly reduced in alpha ablated animals while starburst ablations do not have an effect

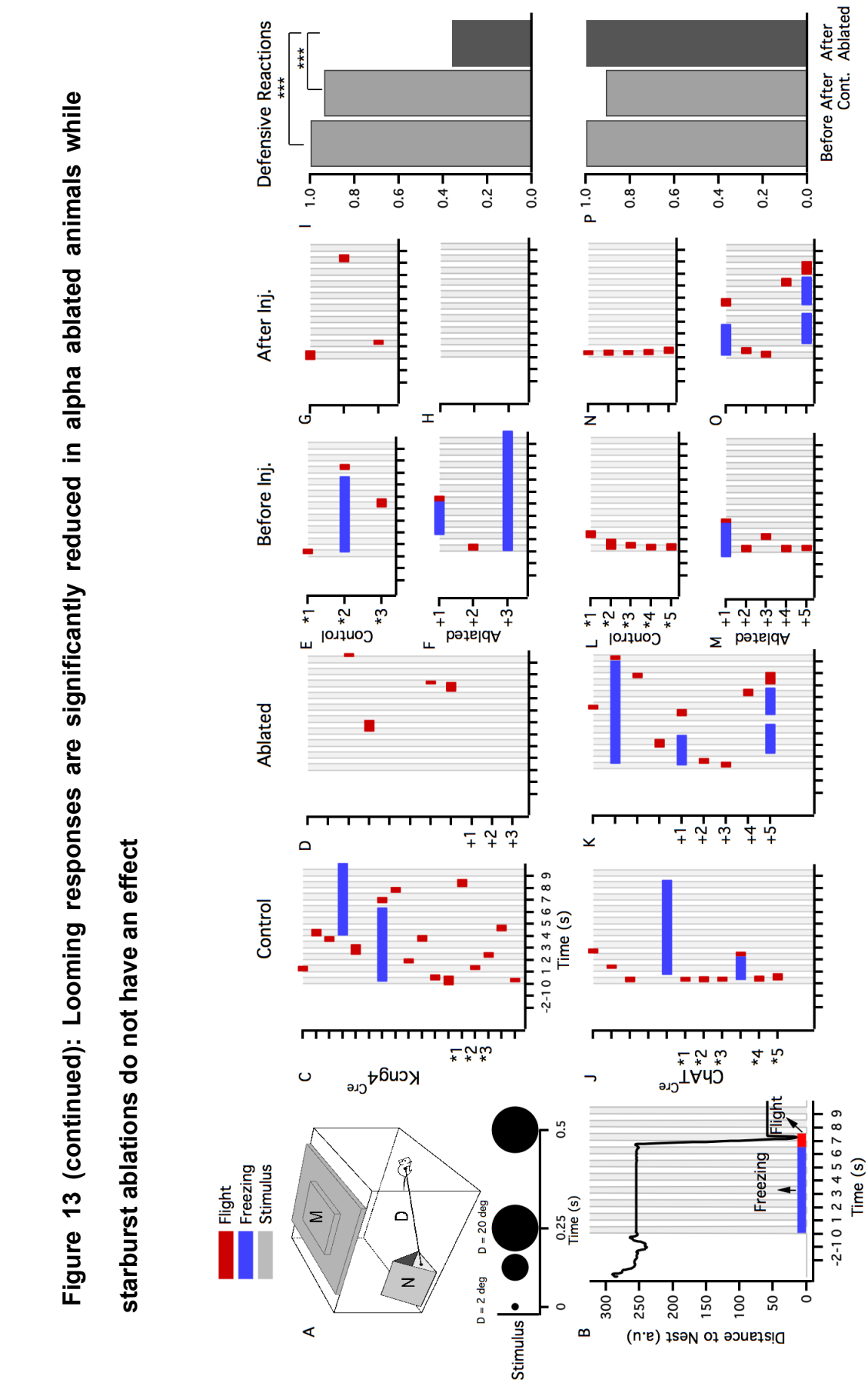
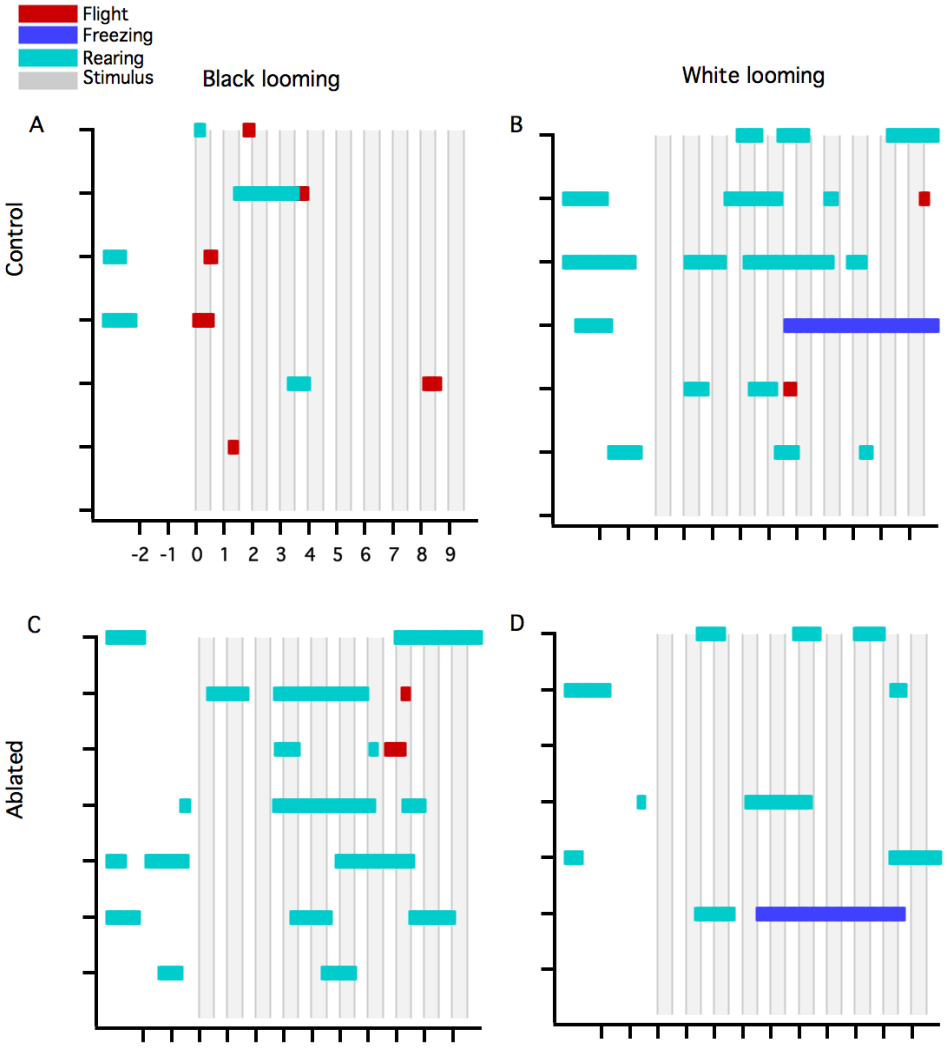


Figure 14: Alpha ablated animals show increased rearing behavior in response to the black looming stimulus but not to the white looming stimulus



Responses of control animals to black looming (A) and white looming stimuli (B). Responses of the alpha ablated animals to black (C) and white looming stimuli (D).

Optokinetic reflex (OKR) is completely abolished in starburst ablated animals while alpha ablated animals have a milder defect

Optokinetic reflex is the movement of the head in response to global motion in order to reduce retinal slip. This was measured in a set-up adapted from previous work (Prusky et al., 2004) where animals are placed on a platform and a rotating sinusoidal grating is shown on a virtual cylinder around them (Figure 15 A). Continuous movements of the head longer than 1 second were automatically extracted and their velocities were calculated (Experimental Procedures). Control animals showed a clear bias of head motion depending on the stimulus direction: they moved their head with positive velocities during counter clockwise stimulus (CCW) and negative velocities during clockwise stimulus (CW) (Examples: Figure 15 D, Figure 16 A, C). A population histogram of all head velocities revealed two well separated distributions in the control animals depending on the stimulus direction (Figure 15 E, I), indicating that animals moved their head mostly in the same direction of the stimulus. In alpha ablated animals there was an overall increase in the number of episodes with the wrong direction (Figure 15 F, pink peaks in the positive velocities) and an overall reduction in the number of head movements (Figure 15 F). To quantify this we calculated a tracking index for each animal, which is defined as (correct – incorrect episodes) / (correct + incorrect episodes). Alpha ablated animals had tracking indices lower than control animals (Figure 15 G) but this did not reach statistical significance. Importantly they were still significantly above 0, which is the chance level. In the starburst ablated population the distribution of head velocities was completely independent of the stimulus direction (Figure 15 J, Figure 16 D). The median tracking index for these

animals was 0, at chance level. The defect in the Kcng4^{Cre} animals may trace back to the ablation of type 5 bipolar cells also expressing Cre in this line since these cells provide input to ON DS RGCs (Yonehara et al., 2013). However at this point we are unable to rule out roles of other cells that were unintentionally ablated or a supplementary role for the alpha cells.

Figure 15: OKR is completely abolished in starburst ablated animals, while alpha ablated animals have a mild effect

Animals are placed on a platform and a rotating sinusoidal grating is shown on the four monitors around the platform (A). Head movements are automatically detected offline by image processing (B). An example trace showing head orientation (C). Example of an extracted head episode is shown in the pink box. Velocity and duration of each episode are calculated for each animal. An example animal is shown in (D). Each circle represents an episode. Episodes during counterclockwise (CCW) stimulus are shown in green and during clockwise (CW) stimulus in pink. Episodes longer than 1 s are considered for further analysis (gray shading). The histograms show the distribution of head velocities for all animals in $Kcng4^{Cre}$ control (E), $Kncg4^{Cre}$ ablated (F), $ChAT^{Cre}$ control (I), $ChAT^{Cre}$ ablated (J) groups. A tracking index is calculated for each animal as (# correct episodes - # incorrect episodes) / (# correct episodes + # incorrect episodes). Population data for each group are shown in box graphs in (G) and (K). Red line is the median, box top and bottom show 25 and 75 percentiles respectively. Whiskers show minimum and maximum. Tracking indices of individual animals that have been tested before and after injection are shown in (H) and (L). Each line represents an animal. Blue is control, red is ablated. *** = $p < 0.005$ based on Mann-Whitney U test for (G) and (K). Before and after tracking indices were compared using paired t-test for equality of two means. * = $p<0.05$.

Figure 15 (continued): OKR is completely abolished in starburst ablated animals, while alpha ablated animals have a mild effect

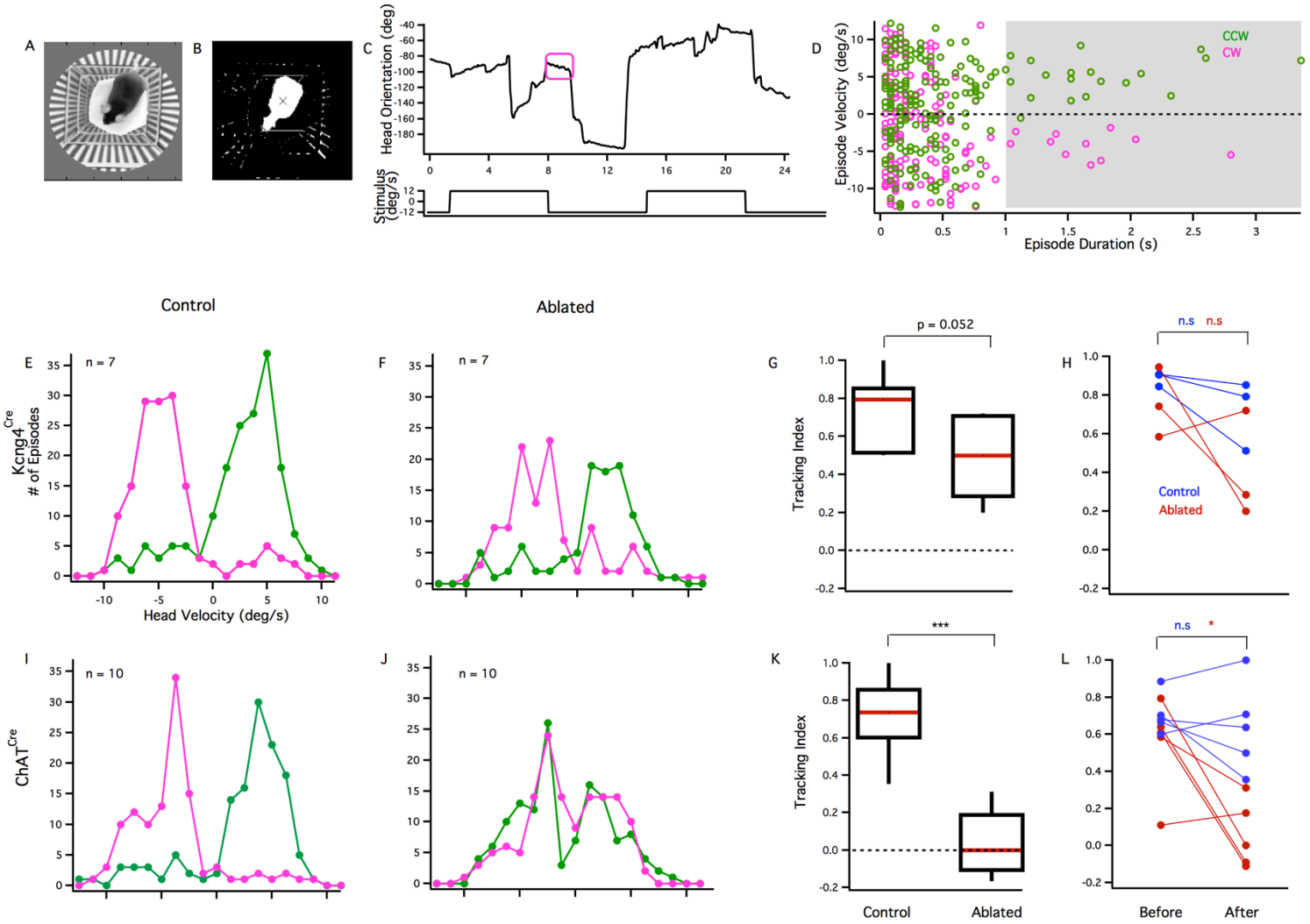
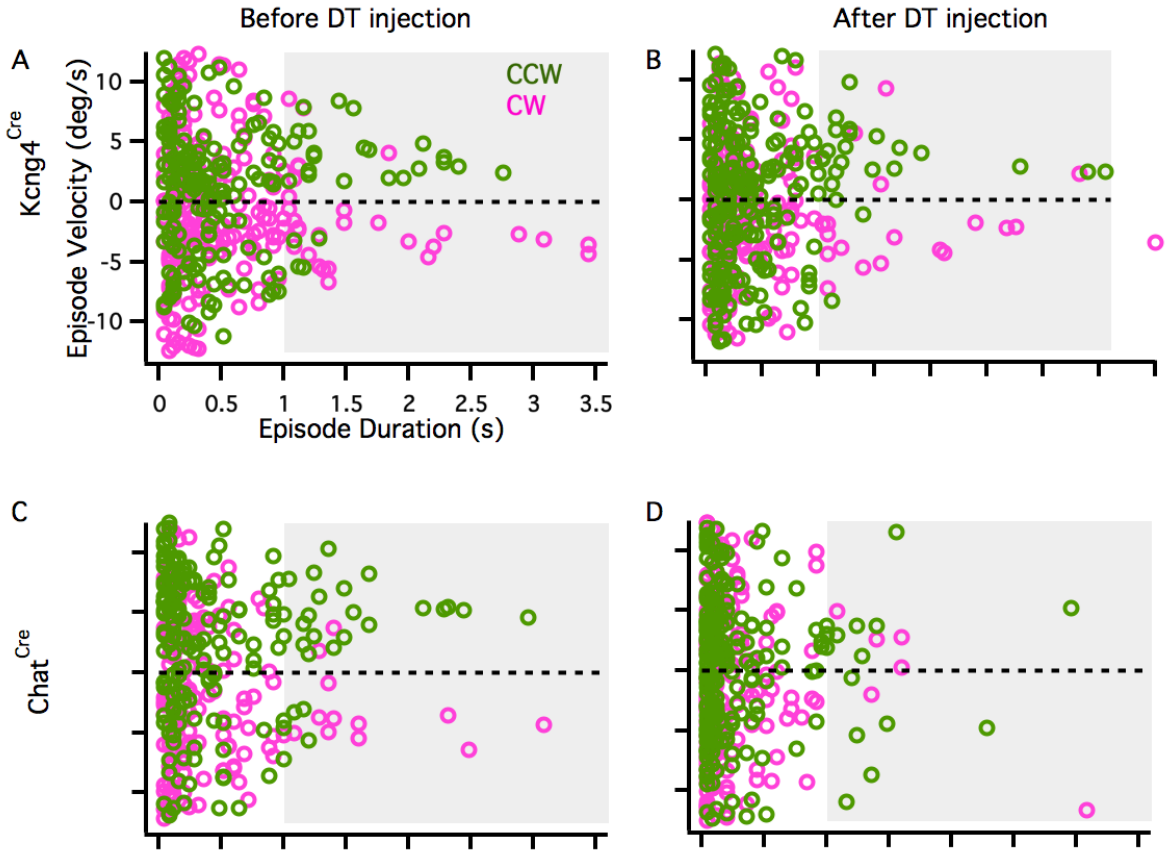


Figure 16: Analysis of head velocities reveals that alpha ablations have a milder effect on the OKR while starburst ablations abolish the reflex completely



Example head episode velocities from two example animals in Kcng4^{Cre} and ChAT^{Cre} strains before and after DT injection. Head velocities of a Kcng4^{Cre} animal before and after DT injection are shown in (A) and (B). Velocities of a ChAT^{Cre} animal before and after DT injection are shown in (C) and (D). Green and pink episodes took place while the stimulus was counterclockwise and clockwise respectively. Gray shading indicates the episodes longer than 1 s that were analyzed for Figure 13.

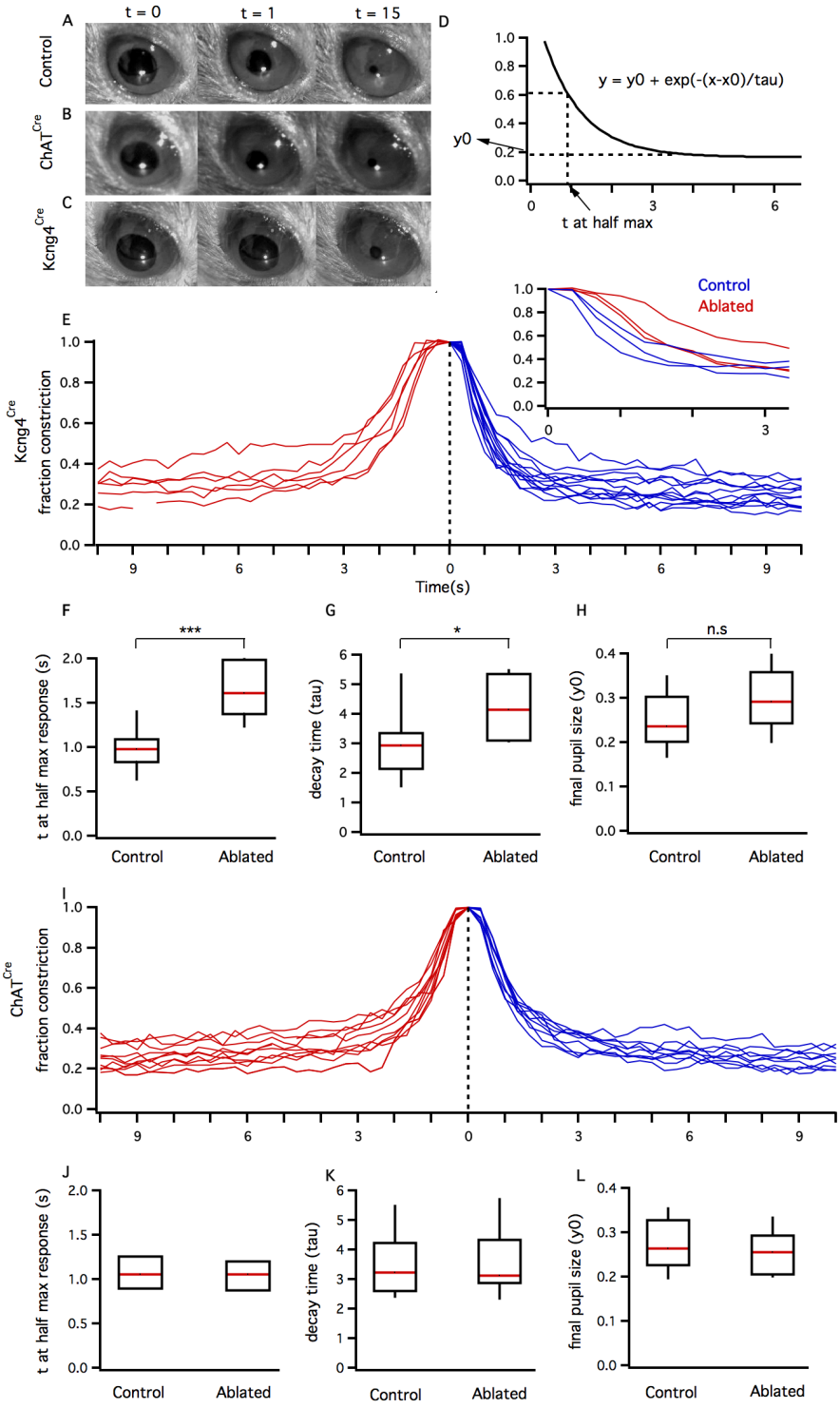
Alpha ablated animals have a delay in the pupillary light reflex, whereas starburst ablated animals have no defect

Animals constrict their pupils in response to bright light. This response was largely intact in both starburst ablated and alpha ablated animals (Figure 17 A-C), however alpha ablated animals showed a delay in constriction as apparent from the relatively larger pupil size 1 s after the light onset (Figure 17 A-C). Normalized pupil sizes are plotted for each animal (Figure 17 E, I). These traces were fit to an exponential and three parameters of the exponential were calculated for each animal: the decay time constant, the final pupil size and the time at which the pupil reached half its maximum constriction (Figure 16 D). Alpha ablated animals show a delay of ~ 0.6 seconds in the pupil constriction, as shown by the increased half-max time (Figure 17 F) and a slower constriction as shown by a higher decay time (Figure 17 G). Starburst ablated animals do not differ from controls in any of the three criteria (Figure 17 I-L).

Figure 17: Alpha ablated animals have a delay in the pupillary light reflex (PLR) while starburst ablated animals have no defect

Example camera frames showing the pupil at t =0, t = 1 and t = 15 s for control (A), starburst ablated (B) and alpha ablated (C) animals. Example exponential fit to the normalized pupil size (D). Fraction constriction for each animal in Kcng4^{Cre} group (E) and ChAT^{Cre} group (I). Traces are normalized to the initial pupil size. Traces from the ablated animals are in red and plotted on the mirrored axis. The inset shows fraction constriction of three Kcng4^{Cre} animals that were tested before (blue) and after ablation (red). T at half max, tau and y0 of the exponential fits to the control and ablated animals are shown in (F), (G) and (H) for Kcng4^{Cre} animals and (J), (K) and (L) for ChAT^{Cre} animals respectively.

Figure 17 (continued): Alpha ablated animals have a delay in the pupillary light reflex (PLR) while starburst ablated animals have no defect



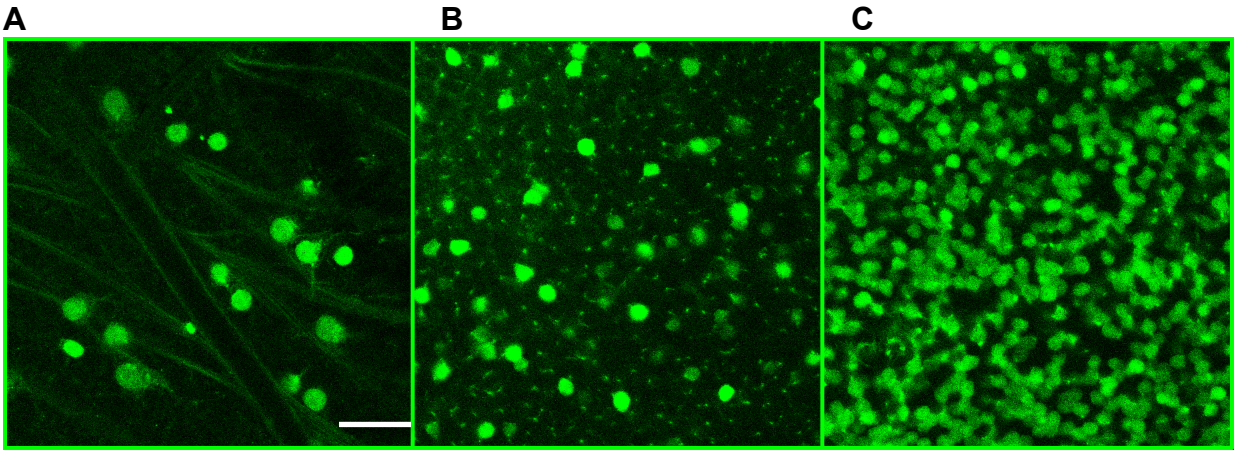
Discussion

We show that two neuron classes labeled by $Chat^{Cre}$ and $Kcng4^{Cre}$ mouse lines have independent roles in three innate behaviors: looming response, OKR, and pupillary light reflex. The $Chat^{Cre}$ line specifically targets the starburst amacrine cells leaving all other cells intact (Figure 9, 11, 12). We show that the direction selectivity of retinal responses supported starburst amacrine cells is essential for OKR but is dispensable for looming avoidance and PLR. The $Kcng4^{Cre}$ line marks alpha cells and type 5 bipolar cells (Duan et al., 2014) as well as other neurons that are still unidentified. Ablation of these cells resulted in a significant decrease in the looming avoidance behavior, a mild defect in the OKR and PLR.

Retinal sections revealed an unexpected loss of cells in the $Kcng4^{Cre}$;iDTR DT+ animals in addition to the alpha and bipolar cells. We consider several possible reasons for this loss and are currently investigating these possibilities. One possibility is a secondary role of the alpha cells in maintaining retinal health perhaps by secreting a growth factor. In this case the retina would be malnourished after the ablation of alpha cells leading to cell death. This effect that would worsen over time. This hypothesis stemmed from the fact that there was a time gap of several months between DT injections and histological analysis in our experiments. We tested this hypothesis by sacrificing the animals 1 week after injections and analyzing retinal sections. We observed the same thinning effect under these conditions, making the possibility of a gradual alpha cell dependent cell loss unlikely. Therefore the defect is more likely due to a direct effect of the ablations.

The second hypothesis we consider is that Cre recombinase expresses in a wider population of cells than just the reported alpha and bipolar cells in the Kcng4^{Cre} line, which can only be revealed with a sensitive reporter. In the work of Duan et al. (Duan et al., 2014; Duan et al., 2015) YFP is expressed under the Thy1 promoter that preferentially expresses in the retinal ganglion cells and some inner retinal neurons like the type 5 bipolars. However in the mice used in our experiments DTR is inserted into the *Gt(ROSA)26Sor* locus, which is known to have a wide expression profile (Zambrowicz et al., 1997). To investigate this possibility we crossed the Kcng4^{Cre} line to a Cre dependent YFP reporter line where YFP is also inserted into the *Gt(ROSA)26Sor* locus (Jackson stock no:007903). The expression pattern of YFP in this line should mimic the expression pattern DTR. Our initial investigation with confocal stacks of whole mount retinas revealed that a large number of bipolar cells and some cells that resemble a single population of amacrine cells were labeled in addition to the alpha cells and bipolar cells (Figure 18). These populations could explain the excessive cell lost in our experiments. We are in the process of co-labeling these cell types with cell specific antibodies in an effort to better understand which cell types were effected by our ablations. To circumvent this problem we injected a Cre-dependent DTR AAV2 virus intraocularly in Kcng4^{Cre} animals. Due to the serotype of this virus, it should only infect the ganglion cells and preferentially affect the alpha cells. However these animals did not show behavioral phenotypes likely due to poor virus efficiency. We are currently investigating the efficiency of the virus using histology on those retinas.

Figure 18: Expression of the Ai3 reporter in the Kcng4^{Cre} line



Confocal images are shown at different depths. (A) Alpha like cells labeled in the ganglion cell layer (z = 0). (B) Population of cells resembling amacrine cells (z = -43μ). (C) Numerous bipolar cells (z = -58μ). Scale bar = 40μ.

A third hypothesis is that the discrepancy of expression between Duan et al. and our retinas results from difference in ages of the animals used in the two studies. Duan et al. used animals at 21 days old, while our animals were 3-4 months old. It is possible that the expression pattern of Cre changes over time, and that there is more expression in the adult. To test this hypothesis we are currently analyzing retinas from 21 days old Kcng4^{Cre} animals that are crossed to the YFP reporter to see if the expression pattern is different than in adults. If this turns out to be the case, we will repeat the experiments by injecting DT at 21 days and testing behavior in adult. At this point we cannot attribute the behavioral defects in the Kcng^{Cre};iDTR DT+ animals solely to the alpha cells, due to the loss of other cells types. Identification of the other cell types that were affected by these ablations will help us better interpret these results. For the rest of the discussion I will focus on cell types that we know are ablated: the starburst amacrine cells, alpha cells and type 5 bipolar cells.

We confirmed that type 5 bipolar cells as well as alpha cells were ablated in the Kcng^{Cre} DT+ retinas. Though we cannot rule out the roles of type 5 bipolar cells in any of the behaviors we tested, we predict that they contribute most heavily to the OKR based on their connections to both ON and ON-OFF DS cells (Yonehara et al., 2013; Duan et al., 2014). This explains the decrease in the tracking indexes of Kcng4^{Cre} DT+ animals (Figure 15 F). The fact that these animals were still able to track the grating accurately indicates that ON DS cells might be getting additional excitatory inputs or that the ON-OFF DS cells might be involved in the OKR. Since the looming avoidance is only triggered by dark looming stimulus that does not activate ON type bipolar cells, the

defect in the Kcng^{Cre} DT+ animals in this assay cannot be attributed to the missing bipolar cells. It is possible that the type 5 bipolars influence the pupillary light reflex, however to our knowledge these cells do not connect to alpha cells (personal communication) or to the M1 type ipRGCs that project to the OPN.

The ON alpha cell that is labeled in the Kcng4^{Cre} line is the M4 type ipRGC (Schmidt et al., 2014), and projects to the core of olivary pretectal nucleus (OPN). We predict that the ~0.6 second delay in the pupil reflex in the Kcng^{Cre} DT+ animals is due to the loss of ON alpha cells. Previously M1 ipRGC has been shown to have a major role in the pupil reflex (Hatori et al., 2008). However the ablation of the M1 type leaves a significant portion (~ 60% constriction) of the response intact under high light conditions (Chen et al., 2011), suggesting that M1 ipRGC is not the sole input for the pupil reflex. Together with the known projections of the M4 type to the core of the OPN, our results suggest that these cells have a role in the rapid initial phase of the pupil reflex. Future studies can test this prediction by looking at the pupil constriction dynamics of the M1 ablated mice. If M4s are indeed responsible for the rapid phase, then one expects to see a rapid but incomplete constriction within the first second of the reflex.

Among the alpha cells, OFF alpha type is a likely candidate for triggering the looming avoidance behavior since the response is triggered by only dark stimuli. The OFF transient alpha cell, also known as the PV-5 cell, has been shown to respond to looming stimuli (Munch et al., 2009). This prediction remains to be tested by a more specific Cre line that only labels the OFF alpha cells. It is important to acknowledge that the OFF

alpha cell also responds to other stimuli that do not trigger avoidance behaviors such as appearance of a dark stationary spot (Munch et al., 2009). Therefore we conclude that even if the alpha cell is necessary to trigger the looming avoidance responses, it might not be sufficient.

Ablation of starburst cells have been previously shown to abolish direction selectivity in DS RGCs as well as the optokinetic eye movements (Yoshida et al., 2001). Our results with the OKR confirm these results. Despite lack of directional signals from the retina the animals had no difficulty sensing and responding to a looming stimulus in starburst ablated animals. Furthermore the directional motion signals from the DS cells are not sufficient to evoke avoidance responses in alpha ablated animals. Altogether these results suggest that direction selectivity is nor necessary nor sufficient to evoke looming avoidance responses. On the other hand ablation of direction selectivity completely eliminates the OKR while ablating almost 50% of the cells in the Kcng4^{Cre} line, though inadvertenetly, has a much milder effect, which indicates the specific reliance of the OKR to the starburst amacrine cells. The behavioral role of direction selectivity in the ON-OFF DS cells remain to be illuminated by future studies.

Kcgn4^{Cre} DT+ animals showed significant loss of the looming responses. The animals that did retain this response had a significant number of alpha cells remaining as revealed by histology (data not shown), suggesting the remaining responses are likely to be due to the remaining cells in the retinas of these animals. Interestingly the animals responded to the black looming spot with increased rearing and to the white looming

with decreased rearing (Figure 14). One explanation is that segregated processing of light and dark information in the retina is preserved in the downstream regions and is used to trigger distinct behaviors. In this experiment we are ablating both ON and OFF types of alpha cells. It is possible that the ON alpha channel predominantly triggers non-defensive postures like rearing and the OFF alpha triggers defensive postures like flight and freezing. In the absence of the OFF alpha cell the dark looming stimulus is sensed by other types of OFF type RGCs that do not respond differentially to looming and trigger rearing behavior. This is consistent with the proposed model in Figure 8.

Finally we observed noticeable differences in the baseline looming responses of $Kcng4^{Cre}$ and $ChAT^{Cre}$ animals. $ChAT^{Cre}$ animals responded to the stimulus with a lower latency than Kcng4Cre animals overall even without the injection of DT (Figure 13 C, J). This difference could be due to different genetic backgrounds. The $ChAT^{Cre}$ a strain has been outcrossed more times than $Kcng4^{Cre}$, and therefore has a more uniform genetic background. This points to the importance of inter-strain differences that could give rise to behavioral variability and highlights the importance of internal controls within the same strain and within the same animal whenever possible.

Experimental Procedures

Mice and Diphtheria Toxin Injections

Kcng4^{Cre} (Duan et al., 2014) and ChAT^{Cre} mice were crossed to mice that express Cre-inducible diphtheria toxin receptor (iDTR) (Jackson Labs stock no: 007900) to yield animals that were heterozygotes for both Cre and iDTR for each strain. The progeny of this cross were injected with diphtheria toxin (1.76 ng/µl.) intraocularly in each eye, twice with a delay of two days. For some of the ChAT^{Cre};iDTR animals the concentration of DT was halved due to concerns about leakage of DT to the brain, which might affect other cholinergic neurons. For these retinas the efficiency of the ablations remained high (data not shown) and behaviors were equally affected as in the group that got the full dose. Both males and females were injected at ages of 3-6 months and tested 2-8 weeks after the injection. Animals in the control groups either received saline injection or received DT injection and did not carry the Cre transgene. We did not observe behavioral differences between the two conditions, thus grouped them together as controls.

Immunohistochemistry

Whole mount retinas were fixed in 4 % PFA for 2 hrs at 4C, then blocked in 5 % Donkey Serum and 1% Triton in PBS for two hours at room temperature. Then they were incubated in primary antibody mixture (primary antibody + 5% Donkey Serum + 0.5% Triton in PBS) for five days at 4C followed by PBS washes and incubation in secondary antibody mixture (secondary antibody + 5% Donkey Serum +0.5% Triton in PBS) for 2 hours at room temperature. They were then mounted on filter paper and covered with

Prolong Gold (Invitrogen) mounting reagent. They were imaged with LSM 710 Zeiss Confocal Microscope. Primary antibodies used were goat anti-osteopontin (R&D systems 1:200), goat anti-ChAT (Milipore, 1:400), rabbit anti-SATB1 (Millipore 1: 5000), mouse anti-smi 32 (Covence antibodies). Secondary antibodies were all from Life Technologies and were used at 1:1000. Neurotrace 530-615 was used to label cell bodies (1:20, Life Technologies). Osteopontin was preferred as an alpha cell marker over the classically used SMI-32 because of its clean labeling of cell bodies (Figure 1). SMI-32 was used to assess the effect of the starburst ablations on alpha cells because the ChAT and osteopontin antibodies are both goat-derived and cannot be used simultaneously. Cells were counted from whole images of retinas and divided by the total area of the retina counted. For $ChAT^{Cre}$ animals, cells in both inner plexiform layer (INL) and ganglion cell layer (GCL) were counted.

Behavioral Testing

Looming Avoidance

Looming Avoidance was tested as described before (Yilmaz and Meister, 2013). Briefly the mice are placed in a behavioral arena 48 cm wide x 48 cm deep x 30 cm high, which has an opaque nest in the corner and a monitor on the top. The looming stimulus is an expanding black disc that goes from 2 to 20 degrees in 0.25 seconds and repeats ten times. The stimulus comes on after mice acclimate to the arena for ten minutes. The behaviors are recorded with a 30 Hz camera. Position and speed of the animal is extracted offline with a custom written MATLAB code. Freezing is defined as the

episodes of 2 s or more where the velocity of the mouse is less than 15% of its average value over the 10 second interval prior to the stimulus onset. Flight is defined as episodes where the velocity of the mouse is greater than 4 times the average velocity over the same 10 second interval as above and the final position of the mouse is in the nest.

Optokinetic Reflex (OKR)

The animals were placed on a 15 cm high platform placed inside a transparent cylinder, which prevented the animals from jumping off the platform and minimized large body movements. A virtual rotating drum was shown on the four monitors surrounding the animal (Prusky et al., 2004), (Figure 15A). The grating had a spatial frequency of 0.12 cpd and rotated at 12 deg/s. It reversed direction of motion every 6 seconds for 4 minutes. Each animal was tested twice, for a total of 8 minutes. The mouse was filmed at 25 fps with a top camera. The movies were processed offline with custom written MATLAB code to extract head orientation and velocity (Figure 15 B, C). Roughly, the mouse was extracted from each frame of the movie using background subtraction. The nose was located as the farthest point from the center of mass of the mouse, and head position was calculated as center of mass of the 150 pixel diameter circle of which the nose is the center. Head orientation is the angle between the head position and nose position (Kretschmer et al., 2013). Episodes of head movements are defined as continuous segments with velocity equal or lower than the stimulus velocity (Figure 15 B,C). Plotting velocity as a function duration for all episodes in wild type animals reveals

a clear separation of episode velocities around zero based on stimulus direction for episodes longer than 1 s. Episodes that happened during counter-clockwise rotation of the stimulus (CCW) have positive velocities and episodes that happen during the clockwise rotation (CW) of the stimulus have negative velocities (e.g. Figure 15 D, Figure 16 A, C). For the rest of the analysis we used only episodes that are longer than 1 s to eliminate the jerky head movements that are not stimulus-related. Tracking index for each animal was calculated as (# correct - # incorrect episodes) / (# correct + # incorrect episodes). A correct episode has a velocity that matches the direction of the stimulus velocity and an incorrect episode has a velocity opposite the direction of the stimulus velocity.

Pupillary Light Reflex

The animals were placed in a restraining device after being dark-adapted for at least an hour. One eye was illuminated with an infrared LED and was being filmed at 30 fps while a flash-light (~1500 lux) was shined on the opposite eye. Pupil size was measured manually every 10 frames from the movie offline. Pupil size was normalized to the initial size and was fit with an exponential y = y0 + exp(-(x-x0)/tau). Fit coefficients y0 and tau and time at half max response (1 – ((1-y0)/2)) were obtained for each fit. Time at half response was calculated as x = x0 – tau*log ((1-y0)/2). Curve fitting functions of Igor Pro was used for this analysis.

Chapter 3: Air righting reflex of mice: The effects of visual cues and age

Introduction

The air-righting reflex during a free fall allows animals to turn from supine (abdomen up) to prone position (abdomen down) so as to land on their feet. In rats it has been described as follows:

> ***When dropped from supine position, the animal immediately ventriflexed the body, then twisted the head and the forequarter almost simultaneously (laterally turned), slightly later turned the hindquarter and took the landing posture*** (Yan et al., 2010)***.***

This reflex has been documented in many animals includind rats, cats and rabbits (Yan et al., 2010; Schonfelder, 1984; Muller and Weed, 1916). We asked (1) Do mice have air-righting reflexes? (2) If they do, do they use visual cues to right themselves?

Results

Mice have air-righting reflexes

To answer the first question we designed a set-up where the mouse was held in supine position with brief suction on a vacuum pipe (Figure 19 A, B, Experimental Procedures). It was released on a well-padded cushion upon turning off the vacuum. During the fall the mouse was videotaped with a high-speed camera (Experimental Procedures). Front paw angle and body angle were measured from the movies at regular intervals during the fall (Figure 19 C, D, Experimental Procedures). Inspection of the movies showed that mice righted themselves immediately after being released in a manner very similar to what has been described for rats (Yan et al., 2010). They first ventriflex their body, then turn their head and front paws followed by their hindpaws (Figure 20). The tail rotated first opposite to body rotation until the body was in prone position, then it either stopped or rotated to the opposite direction halt the body's rotation (Figure 21).

To quantify this behavior we dropped mice from the platform and measured the angle of their paws and pitch angle of the body at each position during the fall as a proxy for whether they righted or not (Figure 19 C, D). If the mouse is in supine position the angle of the paw would be between 0-180 degrees, if the mouse is righted, then it would be between 270-360 degrees. For the body pitch, initial body angle was set as 180 degrees and body angle was measured like the paw during the fall (Figure 19 C,D). Body angles between 0-180 degrees suggest a "butt-down" position while angles 180-

360 suggest a “head-down” position. A body angle close to 0, 180 or 360 indicates a horizontal orientation while a 90 or 270 indicates a vertical orientation.

All mice (n=13) turned their bodies from supine to prone position immediately after being released and maintained this position throughout the fall (Figure 22 A). This was evident from the paw angles at the first position, where the paws were reliably visible. All paw angles were larger than 180 degrees meaning none of the animals were facing up at this point. At the next position the paws converged even further around 270 degrees and stayed that way for the remainder of the fall.

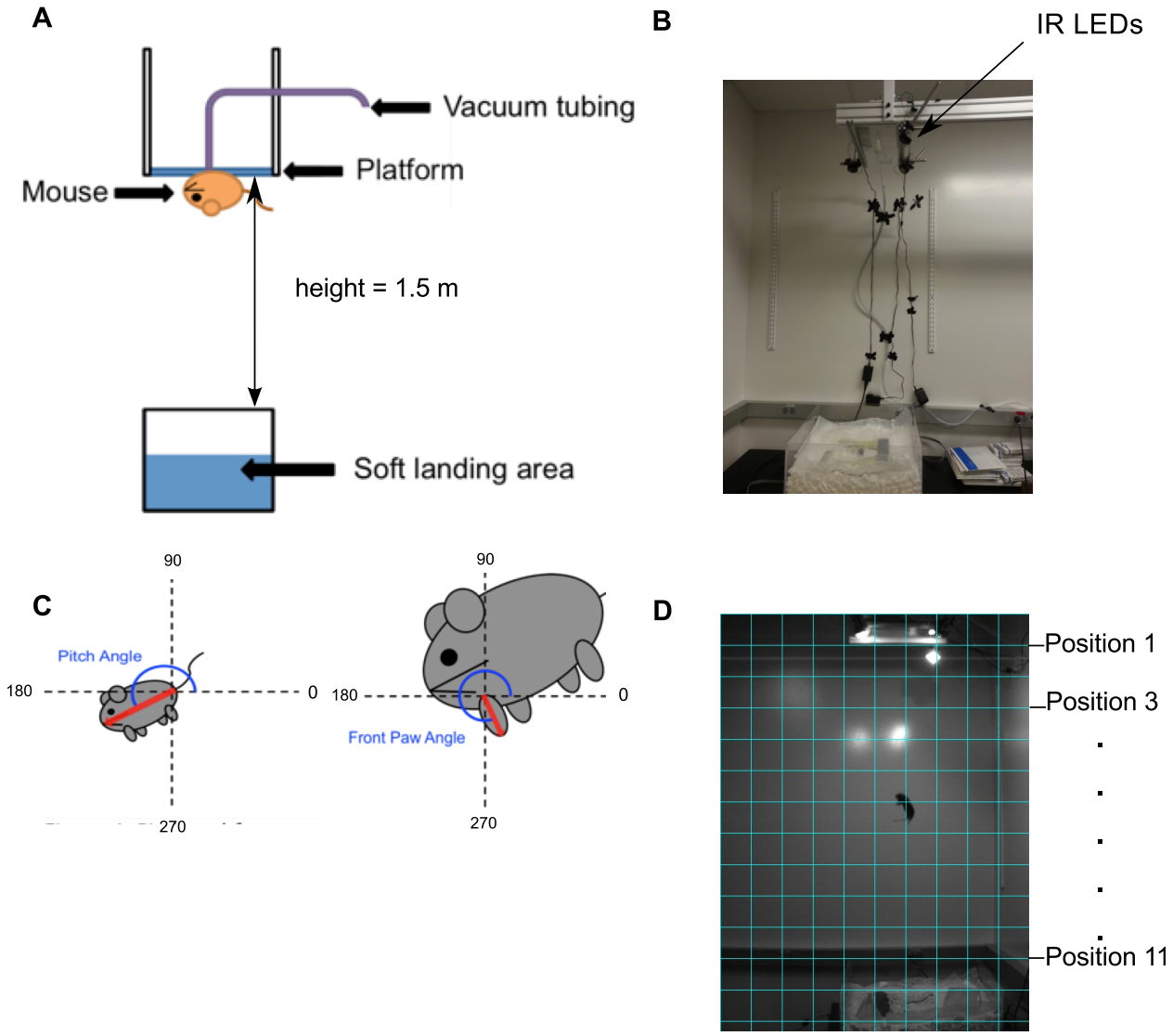


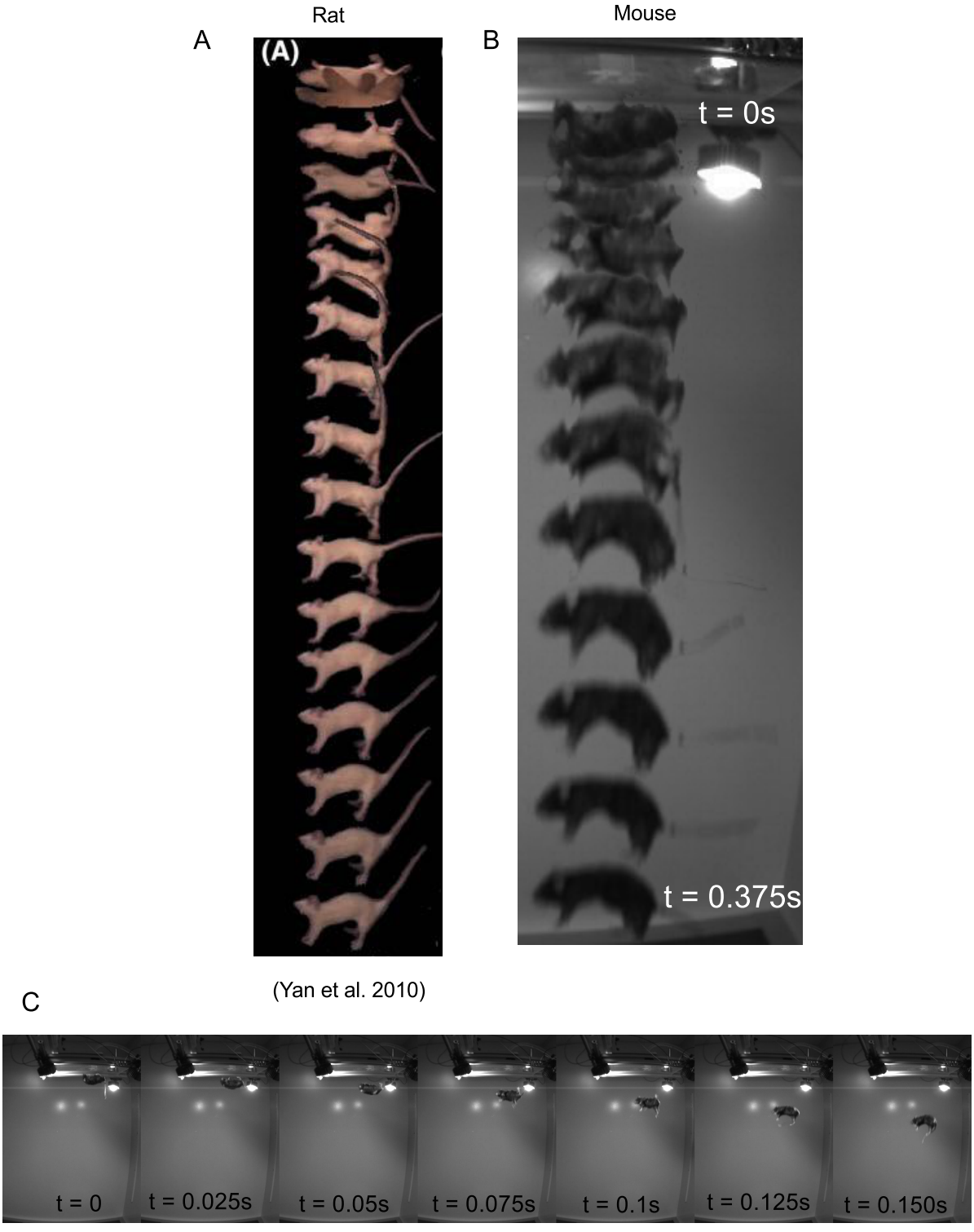
Figure 19: Measuring air-righting reflex in mice

(A) Schematic of the set-up. (B) Photograph of the real set-up. IR LEDs are marked. (C) Definition of the front paw angle and pitch angle. (D) Example video frame from the camera. The grid is overlaid on each frame and is used to normalize the falling distance. The angles were measured at each horizontal line (fall positions) of the grid from the platform to the floor.

Figure 20: Mice have an air-righting reflex similar to rats

(A) Figure from Yan et al (Yan et al., 2010) exemplifying the air righting reflex in rats. (B) Every fifth frame (1/40 seconds) of an example mouse movie is superimposed as a comparison to the rat reflex. The stereotypical ventriflexing of the body followed by rotation of the front and back paws are apparent in both species. (C) Frames from the initial part of the fall in (B) are shown in a horizontal position to better demonstrate the reflex.

Figure 20 (continued): Mice have an air-righting reflex similar to rats



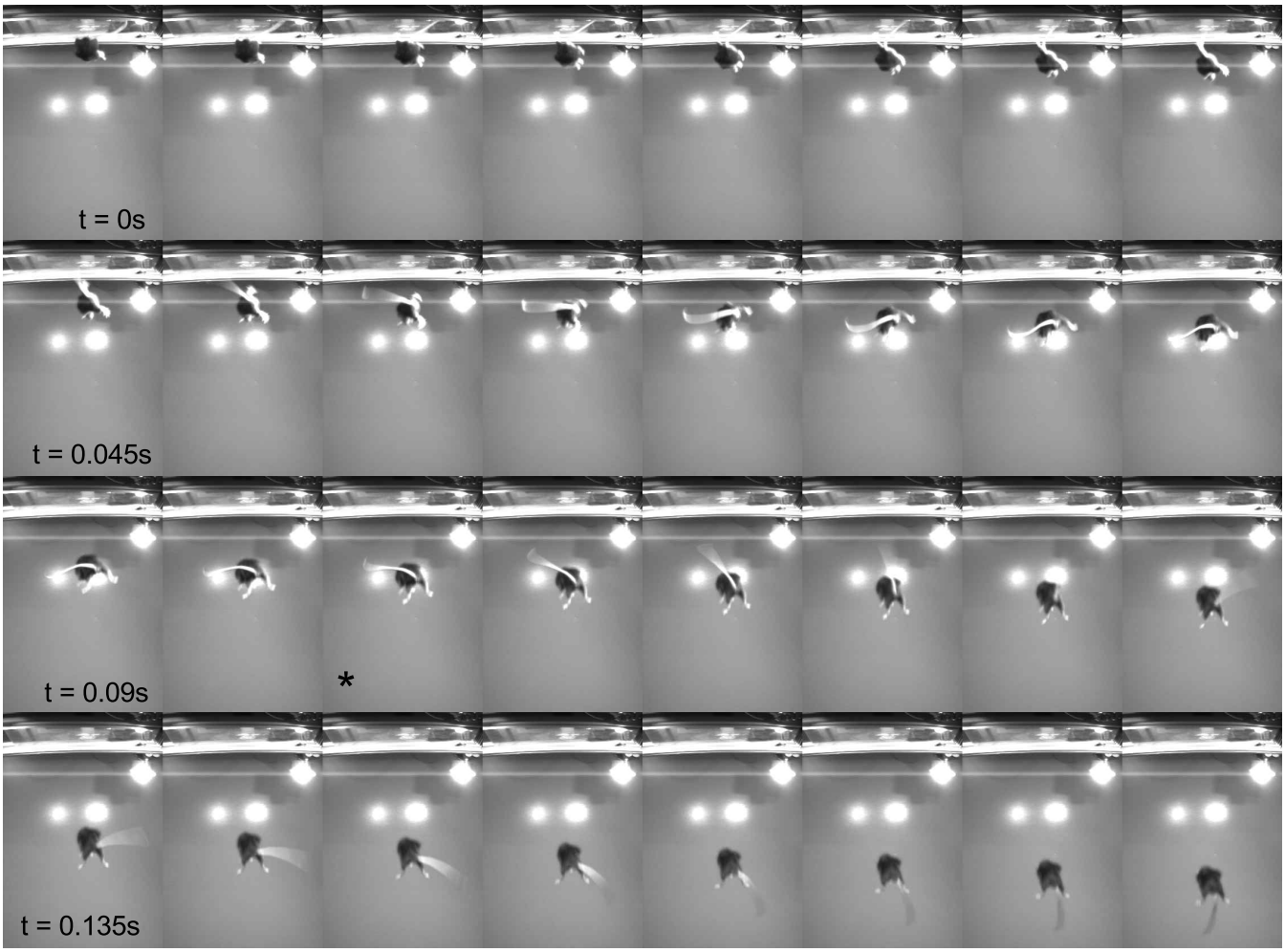


Figure 21: Tail movement during the air-righting reflex

The mouse is placed with hind end facing the camera to capture tail rotation. Each frame is 1/200th of a second. The asterisk indicates the frame where the direction of tail rotation reversed counter-clockwise to clockwise.

The body pitch was slightly more variable than the orientation of the paws. While most animals maintained a horizontal posture slightly head down (~ 200 degrees, Figure 22 C), some animals rotated in the air landing in more vertical positions (~ 270 or 90 degrees).

Air-righting is largely intact in the absence of visual cues (dark condition)

All animals that were dropped in the light were dropped again in the dark under infrared illumination. When dropped in the dark, all animals still pointed their paws towards the floor immediately and righted themselves (Figure 22 B). The paws were slightly more variable in this condition compared to the light condition (Figure 22 A, B). This increase in variability was also apparent in the body pitch with fewer animals able maintain a horizontal posture near 180 degrees and more animals instead flipping and landing in more vertical postures (Figure 22 C, D). We asked whether mice orient their paws and bodies more precisely in the light condition because they remember the location of the floor and other landmarks in the room rather than using visual cues during the fall. To test this we repeated these experiments, this time turning off the lights while the animal was being fixed to the platform and turning the light on before the release (light condition) and keeping it dark for dark condition. In both these conditions mice showed more variable paw angles and body angles while still retaining the overall righting reflex (Figure 22 E -H). No difference was observed between light and dark conditions. These results suggest that air-righting in mice is largely independent of visual cues during the fall, but visual memory might play a role in a more precise orientation.

Air-righting improves with age

During our initial experiments in the light condition we noticed that some animals had more precise falling patterns than others, regardless of the light condition. A closer inspection revealed that these animals were significantly older (~ 5 months old) than the less precise animals (1.5 months old). We asked whether the reflex improves with age. To answer this question we retested the 1. 5 month animals when they grew to 5 months of age. The animals indeed maintained their paws in the downward facing condition more reliably when they were older, suggesting that the reflex improves with age (Figure 23 A, B).

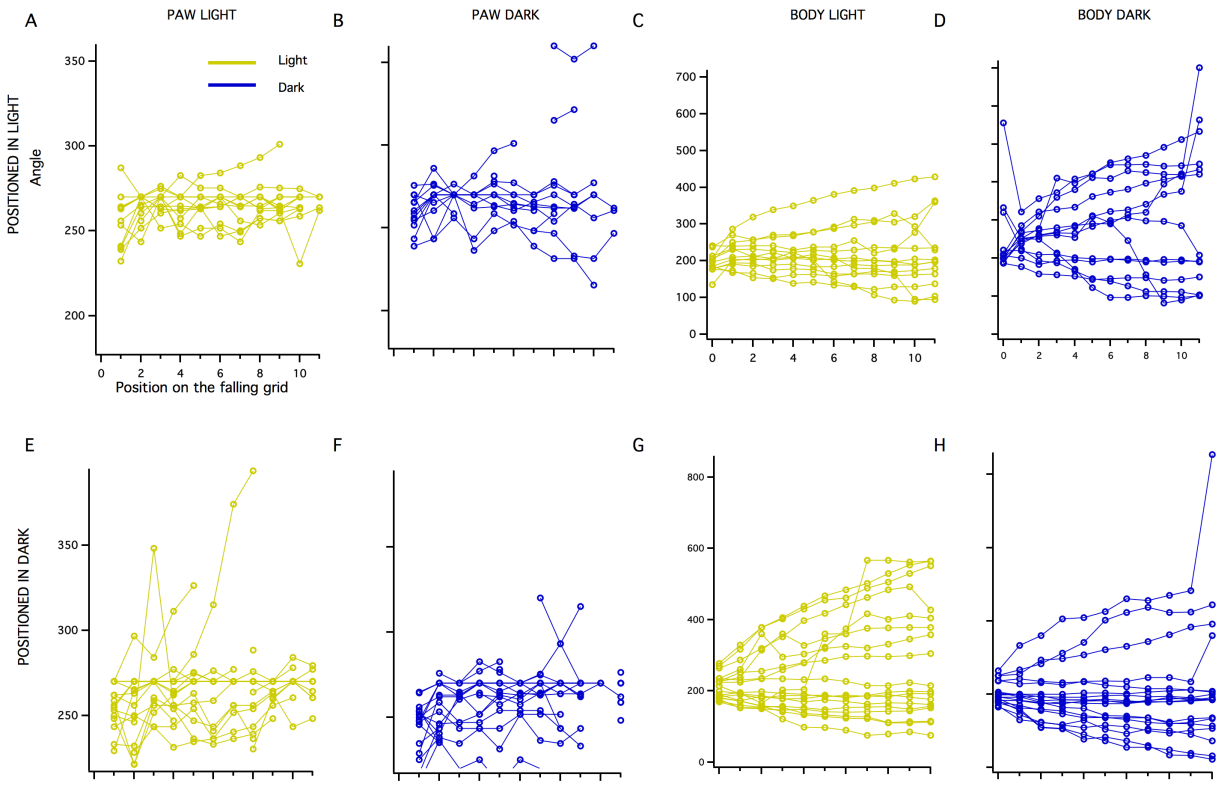


Figure 22: Mice do not use visual cues for air-righting

Paw angles for each mouse are shown in light (A) and dark (B) during a fall (n = 13). Each line represents a mouse. Body angles for the same mice are shown in light and dark (C and D). A separate group of mice (n = 18) were dropped after being fixed to the platform in dark. The paw and body angles are shown for this group (E-H) in light and dark.

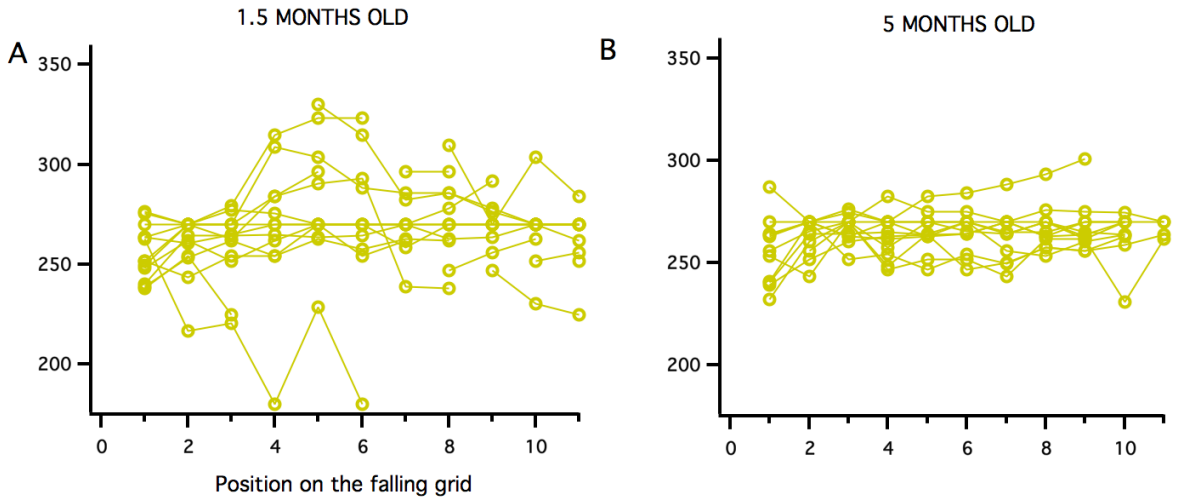


Figure 23: Air righting improves with age in mice

The paw angles of 13 mice are shown when they were dropped at 1.5 month (A) and 5 months (B) of age.

Discussion

In summary our results show that mice like other mammals have a robust air-righting reflex that is largely independent of visual cues and develops with age. This is consistent with the finding that blindfolding a cat has no obvious effect on its air-righting (Watt, 1976). The same study however, showed that labyrinthectomy, a complete removal of semicircular canals as well as the otolith organ, resulted in a complete loss of this reflex while a mere plugging of the semicircular canals had a mild effect, suggesting a major role for the otolith organ. It has also been reported in cats that the air-righting reflex improves with age (Cremieux et al., 1984) consistent with our findings. Our results show parallels with these data from cats and suggest a common non-visual, otolith dependent mechanism for this reflex in mice.

Experimental Procedures

All experiments in this section were performed by Debbie Tsai, a summer undergraduate at Caltech, under my mentorship. Analysis was done Debbie and myself. Each mouse is held in supine position and placed under the “dropping platform” that has a vacuum hole in the middle (Figure 19 A, B). This platform ensures that the mice do not hold on to the vacuum tube and stay in supine position. Mice are dropped at 1.5 meters onto a well-padded cushion when the vacuum is turned off. Both females and males of 1.5 month old (young group) and 5 month old (old group) are used. Each animal is dropped once in light and once in dark condition. In the dark condition the arena is illuminated with infrared lighting for the camera.

Movies were recorded at 200 fps with a high speed camera (AVT Prosilica GE 680). Frames were loaded to ImageJ and a normalizing grid was overlaid on each frame. The front paw angles were manually measured by drawing a line on top of the mouse body and paws and using ImageJ’s measure function for angle measurements. These measurements were taken at every horizontal position of the grid (Figure 19). For measurements of the tail rotation, the mouse was placed with its tail facing the camera on the platform.

Concluding Remarks

In our efforts to link mouse visual behaviors to retinal circuits we discovered

(1) Mice have robust defensive reactions to an overhead looming stimulus (Chapter 1)

(2) Two distinct retinal channels, mediate distinct sets of visual behaviors (Chapter 2).

In the introduction of this thesis I hypothesized that each retinal circuit is dedicated to a specific innate behavior. The findings in Chapter 2 provide evidence in favor of this hypothesis. In this section I will discuss some questions that arise from these findings and ideas for future studies.

Are alpha cells sufficient to trigger the looming behavior?

While we show that the alpha cells could be essential for the looming avoidance responses, our experiments do not rule out roles for other cell types in this behavior. Alpha cells respond to a variety of stimuli that do not trigger defensive behaviors such as uniform darkening or appearance of a dark stationary spot in their receptive fields. This implies neurons in downstream brain regions should do further computations to extract the looming information. How can a downstream neuron differentiate looming from any other dark stimulus? One possibility is that an alpha cell might fire differently to a looming spot than another dark stimulus such as appearance of a stationary spot. In fact the transient OFF alpha cell responds transiently to a dark stationary spot but has a sustained response to the looming spot that lasts as long as the expansion of the stimulus over a variety of speeds (Munch et al., 2009; Pang et al., 2003). Another

possibility is that signals from other cell types that are not by themselves sufficient to trigger behaviors could be contributing to the computation of the looming signal. For instance the animal might use a directional signal to determine that the object is expanding and could ignore it if it is not. However in the absence of directional signals animal could employ a default defensive state. The prediction from this idea would be that the animals show defensive responses to a wide range of stimuli that are not looming. This would be interesting to test in starburst-ablated animals.

Is each RGC type dedicated to one behavior or is it more complicated than that?

Our results show evidence that retinal channels are specialized for specific behaviors. However they don't exclude the possibility that these channels are used in a combinatorial fashion under natural circumstances to regulate a series of complicated behaviors. The mouse's natural environment contains a richer repertoire of visual and non-visual stimuli than our simplistic behavioral arena such as motion signals from other non-predator animals, changing light intensities, food sources, conspecific odors and animal's previous exposure to the stimulus. It is possible that in such a natural environment the other retinal cell types contribute information about other visual cues and influence the animal's decision.

How do mice decide which action to take in response to a visual stimulus? What are the neural correlates of this choice?

How animals decide to flee, freeze or fight in response to threat has been a long-standing question. Some theories defend that the decision is based on distance to threat, while others propose individual differences in animals or availability of escape make a difference (Eilam, 2005). Our results demonstrate that the answer is probably a complicated mix of all the above factors. We show: (1) The parameters of the visual stimulus have a strong influence of on the choice of behavior (Figure 3), (2) Individual animals are capable of exhibiting a number of behaviors in series, which shows that the behavioral choice is flexible (e.g. flight freezing, rearing, tail rattling). Many times flight responses follow immediately after freezing or rearing upon stimulus repetition, and tail rattling was observed in between freezing episodes in the absence of a nest (Figure 4,5). (3) Presence of a hiding place biases decision towards freezing. However ~ 20 % of all animals consistently preferred freezing over flight even in the presence of a nest suggesting other factors such as individual genetic make-up or social status could influence the behavior. (4) Different modes of behaviors are highly stereotyped motor sequences. Animals can switch from one mode to the other in a matter of 10s of milliseconds without the need for prior learning or a secondary stimulus presentation, suggesting the existence of innate motor modules that execute each of these behavioral patterns similar to the “fixed action patterns” described by Konrad Lorenz (Lorenz, 1971). How do these internal and external factors interact with each other to produce defensive modes? Which brain areas are involved? Below I will discuss two existing models previously proposed for the regulation of defensive behaviors and suggest a new working model based on the data presented in this thesis.

Fanselow (Fanselow, 1994) proposes that a group of flight-like behaviors he calls “circa-strike” is mediated by a pathway that involves descending connections from superior colliculus to dorsolateral periaquaductal gray (dlPAG). In his model such behaviors can only be evoked by sensory stimuli, particularly touch. On the other hand freezing is mediated by projections from amygdala to ventral PAG (vPAG), both of which receive inhibition from dlPAG, preventing freezing when flight is needed. Fanselow based his model on the assumption that freezing can only be exhibited after fear conditioning and thus has to go through the amygdala, while flight is only triggered by sensory input through the superior colliculus. His model places the two modes of behaviors on completely parallel pathways with minimal interaction. Therefore it doesn’t account for innate and immediate freezing that we observe in the looming assay and does not explain how the animal can rapidly switch from one defensive mode (freeze) to another (flight).

More recently Mongeau (Mongeau et al., 2003) proposed a model that explains freezing and flight in response to an innately aversive ultrasonic stimulation. In this model the decision of flight or freezing is dependent on activation of different regions within the septum and hypothalamus, that reciprocally inhibit each other, and provide differential excitation to the motor pattern initiators. High excitation of the motor initiators results in a mobile defense, flight, and low excitation results in immobile defense “freezing.” This model provides a plausible explanation for how flight and freezing can switch rapidly via reciprocal inhibitory connections. However the question of where the decision between flight and freezing is made is still unanswered. In addition this model treats freezing as a

behavior resulting from insufficient excitation to the motor areas, rather than a motor program in itself. The freezing reaction in our assay is not merely lack of mobility or sluggish movements but a stereotyped and coordinated pattern of muscle activation that requires its own motor coordination.

I propose a model that incorporates both the fast switching of motor programs as well as the freezing module that is independent of flight (Figure 24). For simplicity I excluded the tail-rattling reaction from the model. According to this model features of the visual stimuli are processed in parallel by different retinal ganglion cells. These features are further processed in retinoreceipient centers such as the superior colliculus (SC) to extract behaviorally relevant features such as looming or upper visual field. Cells sensitive to the looming stimulus have been shown in the SC (Zhao et al., 2014) and this structure is retinotopically organized such that the medial regions respond to the upper visual field (Mrsic-Flogel et al., 2005). Cells specific to behaviorally relevant features feed into a “sensory threat integrator” such that stimulus features with low threat value (white, lower visual field etc.) by definition provide low input to this center, while stimuli with high threat value provide strong input (dark looming). Outputs of the sensory threat integrator get combined with animal’s baseline anxiety to feed into a “perceived threat integrator.” Alternatively cells encoding for distinct visual features can project directly to a perceived threat integrator. This center receives information from sensory and non-sensory areas such as areas regulating animal’s baseline cortisol levels and calculates a threat value. If perceived threat is low, the pre-motor areas that trigger rearing (rearing planning) are activated. Rearing is performed while flight and freezing are inhibited. If the perceived threat is moderate, then freezing pre-motor

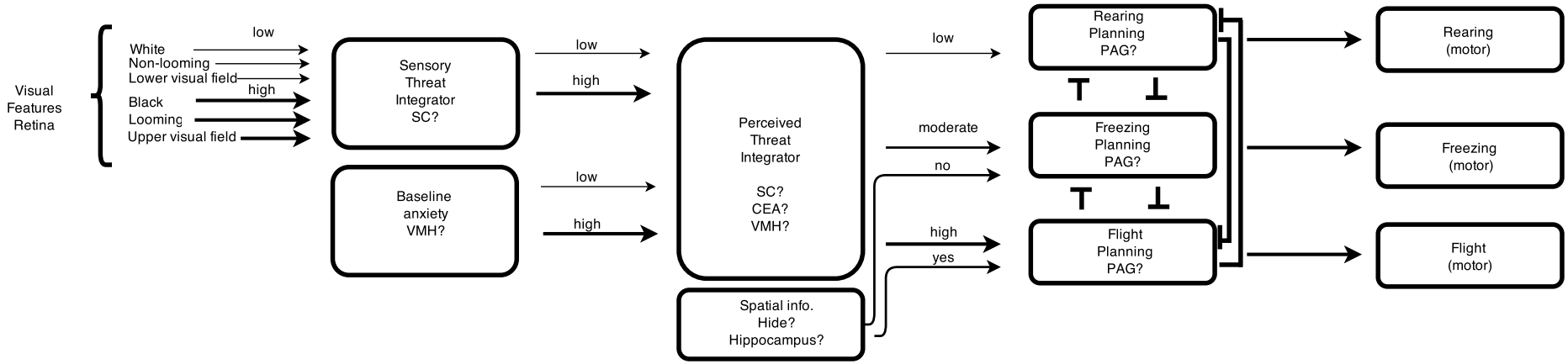
center (freezing planning) is activated, which inhibits rearing and flight. This could happen when a mildly aversive stimulus such as white looming gradually increases the threat value from low to moderate or when the animal has a low baseline anxiety combined with a highly aversive looming stimulus. When the perceived threat level is high, then the flight program is activated, however the flight program requires the presence of a nest to fully execute. In the absence of a nest, flight is generally not activated and in addition “the absence of nest” information provides strong excitation to the freezing program, which takes over. This explains animals switching from freezing to flight as the stimulus keeps repeating thus increasing the threat value. It also explains freezing as the dominant reaction in the absence of a nest.

What are the anatomical correlates of this model? Since the discovery of the looming behavior is relatively recent in mice, there has been little work tying this behavior to any neural correlate. Here I will briefly speculate about the involvement of different regions based on literature from other fear-related assays. A complete discussion for all regions involved in defensive behaviors should be made elsewhere. A good candidate for the perceived threat integrator is the medial nuclei of the hypothalamus since this region has been traditionally been thought as controlling predator defense (Canteras, 2002; Silva et al., 2013). However our work with the Anderson Lab at Caltech revealed that ablations of the SF1 neuron population in the ventromedial hypothalamus (VMH) resulted in defects in a predator avoidance paradigm where the mouse is exposed to a real rat, however left the looming reactions completely intact (Kunwar et al. 2015 (in review)). Our results show that this population is not involved or redundant for the

looming reactions suggesting distinct pathways for different kinds of threat stimuli. A second popular candidate for fear-related behaviors is the amygdala, though this structure has mostly been studied in the context of learned fear (Janak and Tye, 2015). Recent work from the Kim lab at the University of Washington shows that lesions in amygdala have strong effects on the flight responses of rats to a robot predator (http://faculty.washington.edu/jeansokk/Robogator.html). Lateral amygdala has been shown to be involved in integrating sensory cues and regulating predator defenses, however this has always been mediated by the hypothalamic nuclei (Gross and Canteras, 2012). A direct pathway from central amydgala to PAG has been implicated in the context of conditioned freezing responses. It is intriguing to speculate that this pathway could also be involved in innate looming responses, though lesions to the amygdala did not affect footshock triggered immediate freezing (Antoniadis and McDonald, 2001). The intermediate layer of the superior colliculus is another potential site for a "perceived threat integrator." This region is conveniently located close to the visual layers of the superior colliculus and receives input from hypothalamic nuclei involved in defensive behaviors such as VMH, perhaps relaying a information about baseline anxiety (Comoli et al., 2012). Different populations of neurons in the SC have been shown to trigger approach and avoidance behaviors (Sahibzada et al., 1986). Finally the PAG is a traditionally considered an exit relay for defensive responses (Vianna and Brandao, 2003), therefore is a candidate region to plan the execution of different motor modules: consistently stimulation of dorsal PAG triggers escape, ventral PAG triggers freezing (Vianna and Brandao, 2003).

The looming response is a behavioral paradigm where input parameters are easily controlled and motor outputs are easily measured. This model, though overly simple, raises various questions that can be followed by experiments: Are there neurons in the SC that extract behaviorally relevant features of the stimulus (e.g. a polarity invariant looming neuron)? Are there neurons in the SC, CEA or VMH that are visually responsive and whose responses correlate with the sensory threat value of the visual stimulus? Are any of these proposed regions active during the looming evoked defensive behaviors? These are some interesting avenues to pursue to understand the processing of visual threats and control of defensive responses.

Figure 24: A model for transformation of visual inputs to different motor modules



Thick arrows represent strong input, thin arrows represent weak inputs. **T** represents inhibition. SC: Superior Colliculus, CEA: Central Amygdala, VMH: Ventromedial Hypothalamus, PAG: Periaqueductal Gray

Is the looming stimulus aversive to humans? Can these findings benefit humankind?

Human infants respond to a visual display of a looming stimulus with moving back their head and covering their face with their arms (Ball and Tronick, 1971). Interestingly these reactions are not elicited by receding or non-symmetrically expanding displays, reminiscent of mouse reactions. Darwin argued in his famous book “The Expression of Emotions in Man” (Darwin, 1872) that infant reactions shed the most light on the origin of human emotions because they are the simplest and crudest forms of human emotional expression. Based on this idea, infant reactions to looming stimuli may share common mechanisms with mouse looming reactions.

Human behaviors get modified by a great deal of learning during development unlike most other animals. To my knowledge there is no scientific report of human adults responding physically to visual displays of looming objects, though we do encounter this often in IMAC theaters with 3D goggles. However a recent study showed that humans perceive stimuli approaching in space or time as more aversive than receding or static stimuli (e.g looming letters, approaching visit of a disliked cousin) (Hsee et al., 2014). Whether the response is a reflexive limb movement or a form of psychological avoidance, these studies show that the looming stimuli have a high threat value for humans just like for mice. Therefore understanding how visual stimuli can be perceived as threatening in mice can shed light on processing of innately aversive stimuli in humans and help guide therapies for phobias.

On the behavioral output side, humans' reports of their probable reactions to scenarios of various kinds of threats include freezing and flight and parallel the reactions of animals (Blanchard et al., 2001). Further research aiming to understand the underlying principles of these behaviors as well as the underlying neural circuits in mice can shed light into the neural mechanism of similar human reactions. It can enhance our understanding of the pathological defensive reactions and help create new therapies to anxiety disorders such as generalized anxiety disorder (GAD) or post-traumatic stress disorder (PTSD).

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