A Novel Naturalistic Paradigm to Study Aerial Predator-Induced Fear

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Abstract

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Whether an animal is carnivorous or herbivorous, the need to exploit any foraging advantage while mitigating all risks is a major evolutionary pressure. The rat, although a small prey animal, has been able to employ these behaviors to adapt in nearly any environment and become the second most successful mammal on Earth. Historically to study fear behavior and avoidance in the rat, laboratories have utilized Classical Conditioning involving an electrical shock (unconditional stimulus) paired with a tone (conditional stimulus). While this has been proven to be a useful paradigm in understanding learned behaviors and their neural underpinnings, it cannot fully explain the success the rat has had. Multiple innate behaviors have been observed in the rat in response to naturally occurring stimuli, and many of these behaviors are designed to detect and evade predators. A well observed predator avoidance behavior in rodents is freezing or fleeing to an aerial predator or looming stimulus. To further explore this innate fear of aerial predators the present study designed a large arena in which the rat can forage for food and eventually encounters an attacking model owl. All rats immediately fled from the plunging owl and remained in the nest area where other fear behaviors were observed. To begin to understand the neural correlates involved in the observed behavior the basolateral amygdala (BLA) was temporarily inactivated with muscimol. With the BLA inactivated no fear responses were seen and the rat efficiently retrieved the pellet during the owl encounter. This novel paradigm provides a new way to investigate innate defense mechanisms rats use to avoid predation.

Introduction

For nearly all organisms, exploration of the environment and successful foraging determines the survival of that species (Kramer, 2001, Stephens & Krebs, 1986). Specifically the rat is continuously searching for novel sources of food and water near its established nest (Barnett, 1963), and depending on the environment it's in, risks facing a variety of terrestrial and aerial predators. As motivation for resources increases, so too does the distance traveled and mortality threat due to predation (Pellman & Kim, 2016). Threat detection is a strong evolutionary pressure placed on the rat that has entrusted it with multiple ways to sense a nearby predator and innate responses to evade death (Tiffany et al., 1979; Wallace et al., 2013; Litvin et al., 2007). Aerial predators, such as owls, circumvent most of these defenses except for the visual system (Clark, 1975), which has led the rodent to develop sensitivity to looming stimuli and exhibit freezing or escape behavior in response (Pongracz & Altbacker, 2000). For example, a field study conducted found that wild rats chose to feed from trays filled with seed placed in an area with vertical cover compared to an area with open overhead space, even though this leaves the rat vulnerable to terrestrial predator attack. Looming-defense responses (LDR) have been replicated in numerous laboratories using two-dimensional objects displayed above the rodent (Dean et al., 1989; Yilmaz & Meister, 2013), presumably representing an aerial predator showing no learning is necessary for the behavior and allows for the investigation of neuronal responses.

Whether a rodent freezes or flees to a looming stimulus appears to be determined by a multitude of factors including distance from a nest area or shelter and type of overhead stimulus (Yilmaz & Meister, 2013; Wallace et al., 2013; De Franceschi et al., 2016). Yilmaz and Meister (2013) reported that distance from shelter was a significant factor determining fleeing or freezing when exposed to an expanding disc stimulus, where a rodent distant from a shelter would freeze to avoid predator detection in an open area. Alternatively it would become advantageous for the rodent to flee to an adjacent shelter, even if it allows for detection by a predator in the process. Yilmaz and Meister also found the mice did not generalize to the same stimulus presented on the sides of the behavioral arena or to similar changing shapes. De Franceschi et al. (2016) found that the type of overhead stimulus represented the distance of a predator and corresponded to an appropriate behavioral response from the mouse. An expanding black disk representing an immediate threat elicited a fleeing response while a sweeping black disc representing a distal predator produced a freezing responses. Limitations impacting these studies include the utilization of screens mounted to the top of behavioral arenas that are only able to display a two-dimensional image, which may lack the directionality seen during an aerial attack.

A major component of most fear circuit includes the basolateral amygdala (BLA) as a structure necessary for the integration and expression of defensive fear behaviors, as has been shown by decades of research on Classical Conditioning (Maren & Fanselow, 1996; Fendt & Fanselow, 1999; Wilensky et al., 2000). In other studies enlisting a pseudo-predator in a semi-naturalistic foraging task, the BLA was found to be a crucial component of the innate fear circuit, where a lesion or inactivation of the area ablates the fear response (Choi & Kim, 2010; Kim et al., 2013). The predator used in these studies represented a terrestrial predator (Robogator) even though nocturnal foraging rats face the greatest threat from aerial predators (Kortal, 1991).

In the current study we were able to construct a novel foraging apparatus where the rat encounters an owl that plunges downwards while the animals are foraging for food pellets in a large behavioral arena. All rats tested fled from the owl to the nest area where they exhibited other fear behaviors, such as freezing, rearing, and stretching outside the nest area. Rats then had six subsequent days of testing until successful retrieval of the food pellet was achieved through habituation to the Owl. A second group of rats were bilaterally infused with muscimol to temporarily inactivate the BLA that completely abolished any fear response to the owl, and when infused with vehicle the rats were unable to retrieve the pellet. The present study features a model aerial predator experience similar to what a rat would encounter in the wild and provides further evidence of the involvement of the BLA in a wide variety of innate fear behaviors.

Methods

Subjects. 16 male Long-Evans rats purchased from Charles River Laboratory (Washington, USA) were individually housed and maintained on a reverse 12-h light-dark cycle. Animals were placed on a food-depravation schedule with *ad libitum* access to water to reach and maintain 85% of their normal weight. Experiments were conducted during the dark phase of the light cycle. All animal studies and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Amygdala Surgery. Under anesthesia (80 mg/kg ketamine and 5 mg/kg xylazine, i.p.), rats were mounted in a stereotaxic instrument (Kopf) and were chronically implanted with guide cannulae (Plastics One Inc.) bilaterally into the amygdalae. Stereotaxic coordinates for 26-gauge guide cannulae were (referenced from bregma) anteroposterior – 2.5; mediolateral (ML) +- 5.0 and, dorsoventral (DV) +- 7.4 mm. Implanted cannulae were cemented to the skull with 6 anchoring screws. All rats were given 5-7 days of surgical recovery and daily handling and weighing before experimental procedures began.

Foraging Apparatus. A custom-built semi-naturalistic apparatus included a nesting area (29 cm L x 57-66 cm W x 60 cm H) with a gated opening controlled by the experimenter that led to a foraging area (202 cm L x 66-114 cm W). The larger foraging area expanded horizontally at further distances from the nest area to better represent increasing danger while the rat ventures away from the nest. The animal's body-position throughout the entire foraging arena was automatically tracked using the software Anymaze (Stoelting Co.).

Behavioral Procedures. Throughout the entire experimentation period rats were maintained on ~85% normal bodyweight while undergoing successive stages of habituation, baseline foraging training, and Owl testing. Muscimol or ACSF were infused on Owl testing days.

*Habituation***.** Animals were placed in the nesting area for 30 minutes a day for 2 days with 20 food pellets (Bio-Serv; grain-based, 0.5g) and a water-bottle to acclimate to transfer to the experimental room and the nesting area.

*Baseline Foraging Training***.** 24 hours after the 2nd habituation day the animal is placed in the nest area with 2 food pellets and after ~2 minutes the gate to the foraging area is opened so the animal can search for the food pellet placed 25 cm from the nest area opening. When the animal retrieves the food pellet and brings it back to the nest area the gate is closed. Once the animal finished consuming the pellet, foraging trials continued with the food pellet being placed at increasing distances from the nest area (up to 100 cm). Animals underwent 4-6 consecutive days of baseline foraging training consisting of 3 trials per day.

*Owl Encounter Days***.** 24 hours after the last baseline training day the animal was placed in the nest area with 2 food pellets and another food pellet was placed 100 cm away from the nest area. The animal underwent 3 baseline trials then during the first encounter with the Owl the rat had 3 minutes to retrieve the pellet placed 100 cm (long) away from the nest area. The next 3 minute trial had the pellet placed 75 cm (medium) away and on the final trial of the testing day the pellet was 50 cm away from the nest area. When the animal was within 25 cm of the pellet, the Owl was activated automatically through Anymaze. Consecutive day of testing continued until the latency times of all the testing animals returned to near-baseline levels (~5 days).

Drug Infusion. Cannulated animals underwent habituation and baseline foraging training the same as non-cannulated animals. Muscimol-free base (Sigma-Aldrich) dissolved (10mM) in artificial cerebrospinal fluid (ACSF) were microinfused into the amygdala bilaterally by backloading the drugs up a 33-gauge infusion cannula into polyethylene (PE 20) tubing connected to 10-µl Hamilton micro-syringes (Hamilton Company). The infusion cannula protruded 1 mm beyond the guide cannula. 30 minutes prior to the Owl testing infusion cannulae were placed in the guide cannulae to deliver 0.3 µl of muscimol or ACSF at a rate of 0.1 µl/min using a Harvard PHD2000 syringe pump (Harvard Apparatus). The infusion cannulas remained in place for 1 minute after the infusion. Over the next 2 testing days, the infused drug was alternated for each animal.

Owl Apparatus. A life-like owl replica (33" wingspan) was mounted to an air cylinder (3', Bimba) placed at a 45° angle above the foraging area (44"). The air cylinder was controlled using a bidirectional double-solenoid air-valve (IMI Norgren) that was activated automatically through the Anymaze tracking software. Once the Owl was deployed a second signal sent after 2 seconds caused the air cylinder to retract. Once retracted, the Owl was hidden from view to the foraging animal by a blackout curtain.

Histology. At the completion of behavioral testing, animals were overdosed with Buthanesia and perfused intracardially with 0.9% saline followed by 10% buffered formalin. The brains were removed and stored in 10% for 24 hours and then kept in 30% sucrose solution until they sank.

Transverse 50-µm sections were taken through the location of the cannulae, mounted on gelatin-coated slides, and stained with a cresyl violet dye (Figure 6).

Statistical Analysis. To analyze the difference in animal's baseline latencies across testing days a related-sample Wilcoxon-signed rank test was performed between testing day 1 baseline and each subsequent day of testing using the statistical software-package SPSS (IBM).

Results

Simulated attack of plunging owl while foraging leads to immediate flight to nest area

All animals quickly learned to search for pellets in the foraging area of the arena and return to the nesting area up to 100 cm away from the nest area. After 4-6 days of baseline training the Owl was activated, traveling 3 feet in ~1 second, when the rat was within 25 cm of the pellet. During the first day of testing all animals immediately fled from the owl to the nest area where they then exuded multiple fear behaviors (i.e. freezing, stretching outside the nest opening) and no animal was able to retrieve the pellet while multiple animals made no second attempt at retrieving the pellet after the initial Owl attack. After the first 3 minute test was finished the unretrieved pellet was placed 25 cm closer to the nest area (75 cm away) and the animal was given a second attempt. Again, no animal was able to retrieve the pellet and this behavior remained reliable even when the pellet was placed 50 cm away from the nest opening. For the entire group to return to comparable latencies from before the Owl was presented took 5 consecutive days of testing. Most animals first retrieved the pellet from the shortest distance away and starting the next day of testing would venture out to greater lengths in order to retrieve all 3 pellets available during the testing day.

Baseline latencies increase day after first owl exposure

Up until the second day of testing, the baseline performance of all the animals tested was below 20 seconds to retrieve the pellet. On the second day of testing following the first exposure to the Owl, the latency to retrieve the pellet significantly increased when compared to baseline latency on the first day of testing (related-samples Wilcoxon Signed Rank Test, $z=32$, $p<0.05$; figure 4). This effect from the Owl continued through the 3^{rd} day of testing's baseline latencies (related-samples Wilcoxon Signed Rank Test, $z=32$, $p<0.05$; figure 4) and decreased down to comparable levels to initial baseline latencies the same day the majority of rats habituated to the Owl. The significant increase in time to retrieve the pellets during baseline suggests the rats formed a contextual fear memory to the Owl and foraging area, further supported by the extinction of the fear after exploration and retrieval of the pellet.

Reversible inactivation of the BLA attenuates fear response to Owl

To determine whether the expression of fear in response to an overhead predator requires the BLA as has been shown with terrestrial predators, the GABA agonist muscimol was infused into the BLA during Owl testing. After baseline foraging training had been completed (4-6 days) on the first day of testing 30 minutes before the trial began, the animal was infused with 0.3µl of either muscimol or ACSF (0.1µl/min). The muscimol infused animals successfully retrieved the pellet at all distances when confronted with Owl, while the ACSF infused animals failed to retrieve the pellet from any distance. The next day the infused drug from the first day of testing was alternated for each animal. Once again the muscimol infused animals did not show any fear responses, while the ACSF infused animals failed to retrieve the pellet. On the third and final day of testing the rats were infused with the original drug they received during the first Owl test.

The ACSF infused animals once again were not able to retrieve the pellet and the muscimol infused animals showed no fear response to the Owl.

Discussion

In the current study, the innate fear response to aerial predators was successfully replicated in a laboratory setting utilizing a semi-naturalistic foraging paradigm and a plunging owl predator. Following successful foraging training, rats were unable to successfully retrieve a food pellet placed outside of the nest area and required 5 days of subsequent testing in order to achieve habituation to the Owl. The animals were also able to form a contextual fear memory from the Owl encounter after the first day of testing as evidenced by the increase in baseline foraging latencies the second and third days of testing. This finding is of particular interest because up until the first Owl testing day, the rat had thoroughly explored the foraging area with a 100% success rate with no dangerous stimuli presented. So after one day of owl exposure a previously safe foraging area has been recoded as a dangerous space. Other studies on classical conditioning have found that being placed in a familiar 'safe' context or home cage diminishes the effects of fear conditioning (Goode et al., 2015).

When comparing the rat's behavior when confronted with an aerial predator to other studies utilizing a terrestrial predator (Choi & Kim, 2010; Kim et al., 2015; Kim et al., 2013) there is a clear increase in the fear response to the Owl versus the Robogator. Exposing the rat to a hidden aerial predator while foraging provides a more realistic, possibly more intense, predator stimuli than a terrestrial predator representation. Field studies have shown when given the choice between seed trays, nocturnal rats show a preference for trays placed in an area with more vertical foliage coverage than trays placed out in the open (Kotler et al, 1991). Areas with more horizontal foliage cover contribute their own set of risks from terrestrial predators like the fox, snake, and weasel while less vertical coverage allows for predation from owls. Throughout its evolutionary history the rat has developed sensory capabilities to detect predators and behavioral strategies to avoid terrestrial predators, but only relies on its low-quality vision to detect aerial predators. While it has already been shown in laboratory studies that rats innately avoid the scent of a fox (Coryell et al., 2007; Kobayakawa et al., 2007; Fendt et al., 2005) and show LDR's to expanding discs and sweeping bars (Boissy, 1995; Gross & Canteras, 2012; Pongracz & Altbacker, 2000), there has not been any investigation into how rats distinguish between a terrestrial and aerial predator as they appear to display in the wild. Future studies should focus on neuronal populations that discriminate between the two types of threats.

An area involved in the innate fear response to aerial predators is the superior colliculus (SC), which has been shown to be crucial in visually guided behavior, approach-avoid situations, and startle responses (Sprague & Meikle Jr., 1965; Ghandi et al., 2011; Dean et al., 1989; Edwards et al., 2011), and receives direct input from retinal-ganglion cells involved with motion detection, speed, and direction (Weng et al., 2005; Zhang et al., 2015; Munch et al., 2009; Sahibzada et al., 1986). Regarding defense responses from looming stimuli, the BLA may be involved, as shown through projections originating from the SC in both rats, human, and nonhuman primates and terminating in the BLA (Rafal et al., 2015; Linke et al., 1999). Coker-Appiah et al. (2013) reported a strong amygdala activation in humans in response to looming animate threatening stimuli, but not inanimate objects (i.e. guns, knives). A circuit-level response to looming stimuli may be conserved across mammalian species.

Rats and mice are not only able to survive but thrive across every continent (except Antarctica) even though they are constantly under the threat of predation. The innate threat detection and response behaviors the rat uses allows for this global success. Further exploration of this LDR circuit will be useful in analyzing not only strong innate fear behaviors, but anxiety behaviors in a fearful context. The Owl pseudo-predator threat that we have employed in this study allows the investigation of a more dynamic fear setting; one that adds the uncertainty of a hidden predator and the increased danger of an expanding open-area.

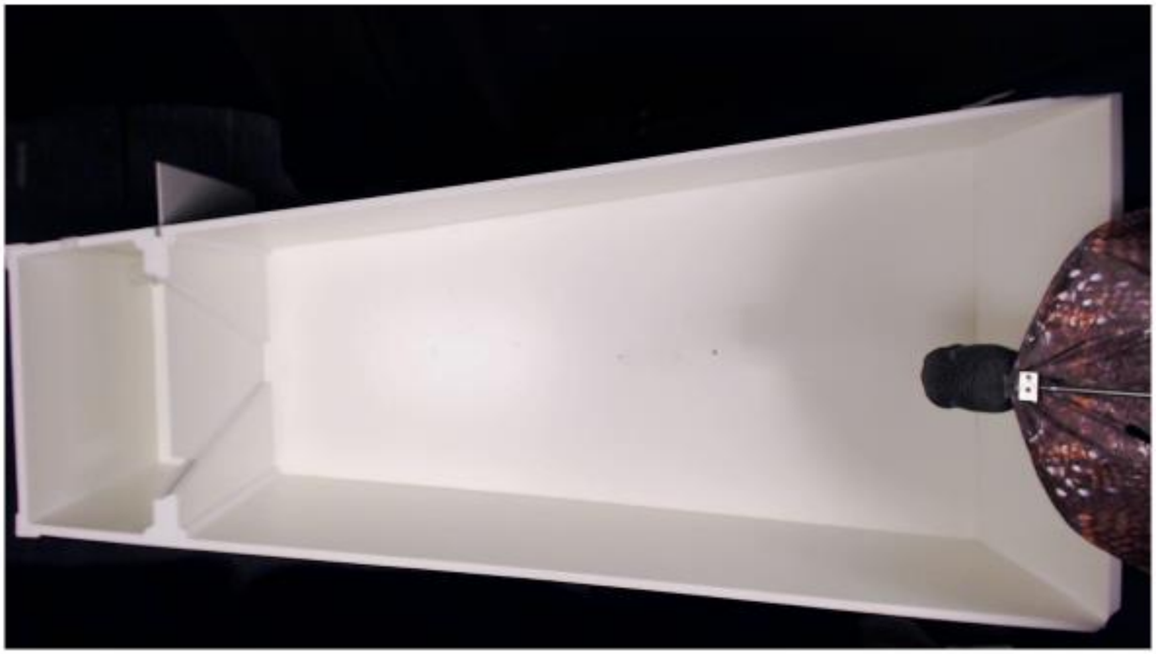

Fig. 1-Entire foraging and nesting arena with owl retracted, but no curtain to conceal it. Nest area: 29 cm long, width expands from 57-66 cm, 60 cm tall. Foraging Area: 209 cm long, width expands from 60 to 114 cm.

Fig. 2-Foraging arena with curtain after rat has encountered owl. Rat is watching outside the nest before making another attempt to consume a food pellet. Owl is retracted and hidden from rat's view behind curtain.

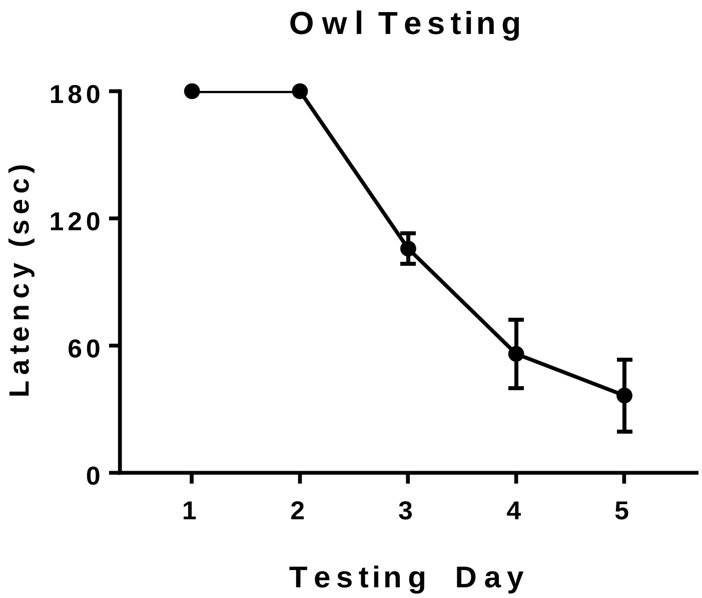

Fig 3. - Latency to retrieve pellet across all Owl testing days. By the third day some rats begin to successfully reach the pellet, but it takes 5 days of testing for latency to drop down to pre-Owl amounts.

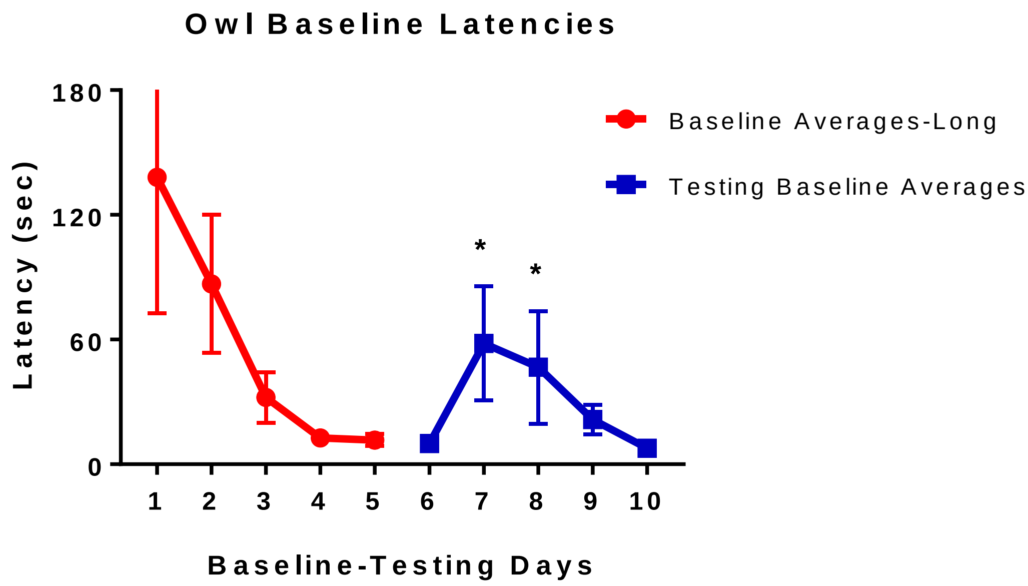

Fig. 4- Baseline latencies during training drop sharply after 5 days of training, indicating ability to quickly forage for food pellet. When the rat is exposed to the owl on day 6 (Testing day 1) there is a significant increase in baseline latencies across the next two days (stats). This may suggest contextual fear conditioning through the representation of a realistic predator. A related-samples Wilcoxon-Signed Rank test was performed between day 6 and all other testing days. Only days 7 and 8 were significant was compared to day 6 (z=32, $p<0.05$; z=32, $p<0.05$).

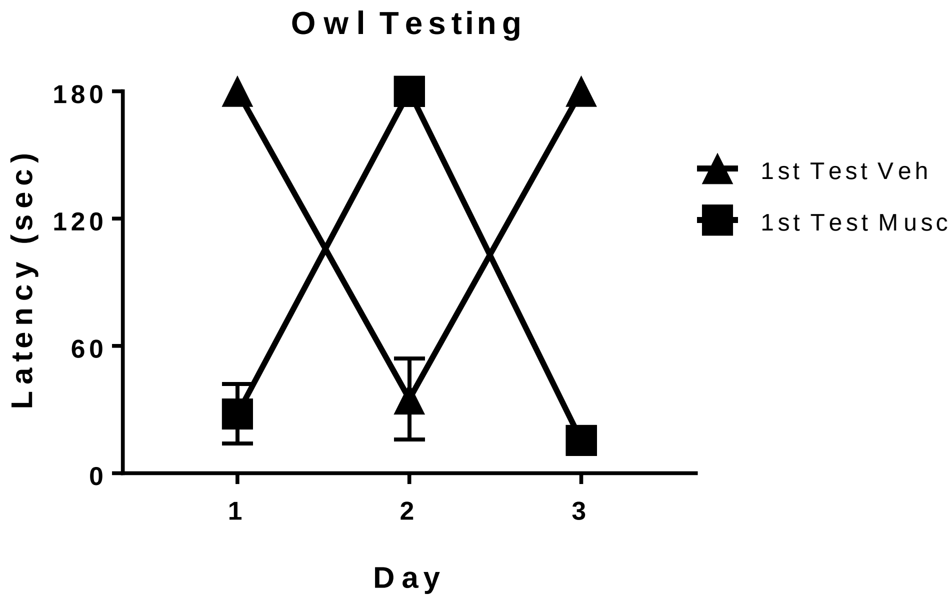

Fig. 5-Latencies during testing for cannulated animals. For both groups muscimol abolished any fear response to the Owl, while ACSF had no effect. The schedule of drug that was infused prior to testing was alternated each day (i.e. 1st day muscimol, 2nd day ACSF, 3rd day muscimol).

Fig. 6-After behavioral testing was completed cannulae placement was confirmed through cresyl violet staining. Infusion cannulae extended 1mm beyond end of guide cannulae. AP: -2.5, ML: +-5.0, DV: +-7.4.

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