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Effects of the Stimulus and Chamber Size on Unlearned Fear Across Development

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Abstract

Predator odors have been found to induce unconditioned fear in adult animals and provide the opportunity to study the mechanisms underlying unlearned and learned fear. Predator threats change across an animal's lifetime, as do abilities that enable the animal to learn or engage in different defensive behaviors. Thus, the objective of this study was to determine the combination of factors that successfully induce unlearned fear to predator odor across development. Infant, juvenile, adolescent, and adult rats were exposed to one of the three odor stimuli (control odor, cat urine, or cat fur) in either a small or large chamber. Though all ages displayed fear-related behavior to cat odors, differences were reflected only in freezing behavior and not, as expected, risk-assessment. Infant and juvenile animals also increased freezing to cat urine compared to the control odor, possibly because these age groups possess limited defensive options to cope with threat and so may respond with freezing to all predator stimuli. Unexpectedly, chamber size had no effect on either freezing or risk-assessment in this study. Once the parameters of unconditioned fear are understood, they can be exploited to develop a learning paradigm to predator odors that could be used in early life.

1. Introduction

The ability of an organism to innately respond appropriately to a severe threat has ultimate survival advantages (Kavaliers and Choleris, 2001). For example, adult rats respond to cat odors by engaging in increased freezing and risk-assessment behaviors (Blanchard et al., 1990; Zangrossi and File, 1992; Dielenberg and McGregor, 2001). The rat may avoid detection by the cat by freezing, while various risk-assessment behaviors such as stretch-attends or flatback approaches allow the rat to monitor a potential threat. Risk-assessment may be a more adaptive response to predator stimuli than freezing in the adult rat, particularly under conditions of low danger when time and energy can be better spent in activities such as caring for young or foraging (Klein et al., 1994; Blanchard et al., 2003a). However, little is known about how developing animals respond to various predator odor cues (but see Muller-Schwarze, 1972; Lyons and Banks, 1982; Moriceau et al., 2004). Specifically, it remains to be determined which cues are the most effective at eliciting a fear response at different ages and what these cues may represent for these age groups.

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Prey response to a predator is also dependent upon the amount of space which separates them, according to Fanselow and Lester's predator imminence model (1988). Predator/prey interactions model fear and anxiety depending on whether or not a threat is immediate or anticipated, and this threat level can be manipulated by changing the type of stimulus used and the space available in the experimental chamber (Blanchard and Blanchard, 1990). If the predator is in close proximity, the prey has little opportunity for escape and thus primarily freezes to decrease conspicuousness. If there exists a greater distance between predator and prey, the prey may engage in risk-assessment or avoidance behaviors. Therefore, space confines affect an animal's behavior by increasing its perceived predation risk due to a lack of defense options and reducing the potential for different coping behaviors (i.e. risk-assessment) to emerge (Ward et al., 1997).

The type of stimuli and its proximity interact to produce a signal of danger, however the exact conditions which direct behaviors in one direction or another are not completely understood (de Oca et al., 2007). Most experimental paradigms using rats and predator odors have more than likely exaggerated fear learning due to the intensity of the stimulus used and small spatial confines of the apparatus. Additionally, unlearned fear behavior to a predator should vary with the age of the respondent. To test the hypothesis that behavioral responses of rats change as a function of these factors, infant, juvenile, adolescent, and adult rats were exposed to either cat fur, cat urine, or a control odor in a small or large chamber. It was hypothesized that all ages would display fear-related behavior to cat odors since these stimuli have been found to be effective in inducing behavioral changes in adult and juvenile rats (McGregor et al., 2002; Blanchard et al., 2003b; Hubbard et al., 2004).

However, it was expected that different age groups would use different strategies to cope with threat. Younger animals, unable to flee as effectively as older animals, were expected to prefer freezing over risk-assessment while older animals should display increased risk-assessment over freezing. It was expected that animals would display an increase in fear-related behavior during exposure to cat fur compared to cat urine and the control odor since cat fur odor may signify a higher level of threat and the actual presence of the predator than a scatological odor cue that may signify that the predator was historically present (Blanchard and Blanchard, 1989). Lastly, based on the predator imminence model (Fanselow and Lester, 1988), it was also predicted that animals exposed to cat odors would respond with heightened levels of freezing behavior in the small chamber and heightened levels of risk-assessment in the large chamber.

2. Materials and Methods

2.1 Animals

All experiments used wild-type Long-Evans hooded rats bred in the animal care facility of Columbia University and housed in standard laboratory cages. Cages were maintained in a colony room with a temperature of 22–24°C and monitored daily in the morning and evening for the presence of newborn pups and the date of birth was considered as post-natal day (PN) 0. Animals were given *ad libitum* access to food and water and exposed to 12h light/dark cycles beginning at 6:00 A.M. each day. Rats were separated from the dam on PN23 but remained with littermates in the same cage. Adolescent and adult rats were kept in cages with one or two same-sex littermates. Rats of four different ages – infant (PN14), juvenile (PN26), adolescent (male: PN35, female: PN45), and adult (PN90–100) were used. For each experimental condition, there was an equal number of female (N=3) and male (N=3) animals to control for sex differences. Additionally, no sex differences in threat-induced freezing have been observed in infant, juvenile, and adult rats (Wiedenmayer and Barr, 1998; Falconer and Galea, 2003). The infant animals were odor-exposed on PN14 and the juvenile animals on PN26. For the adolescent group of animals, 35 day-old females and

45 day-old males were used since adolescence begins earlier in female rats than male rats (Coe et al., 1981). Adolescence in the rat is a variable, protracted period between PN28–42 and contains many transitions (Spear, 2004). More stable than age in determining adolescence is weight (Spear, personal communication) and a conservative estimate was used based on age and weight. Estrus cycle was determined by vaginal swab cytology and only females in diestrus were used to control for changes in behavior (eg. locomotor activity) that occur in females at different points in their cycle. Each animal was tested in only one experimental condition. All tests and treatment procedures were approved by and in accordance with the Institutional Animal Care and Use Committee of Columbia University.

2.2 Apparatus

In order to test odor stimuli for different ages of rats, an acrylic box with a hinged top lid and internal width and height of 15.25×20.25 cm was used (Figure 1). The box contained a movable, solid divider that was adjusted depending on the age of the rat and contextual condition. At each age, both a small chamber (approximately one body length) and a large chamber (3 times the approximate body length) were used. For infant rats, the total length of the small chamber was 7.6 cm and 22.9 cm for the large chamber; juvenile rats, 10.2 cm and 30.5 cm; adolescent rats, 15.2 cm and 45.7 cm; and for adult rats, 22.9 cm and 71.1 cm. The chamber was cleaned with 70 % isopropyl alcohol before each use and paper towel was taped to the floor of the chamber. In the large testing chamber condition, a 3 (across length) $\times$ 2 (across width) grid was drawn on the paper towel in order to measure proximity and activity.

2.3 Odor Stimuli

The odor stimulus was delivered via an odor box that affixed over the odor port on the outside of the apparatus (see Figure 1). The stimulus consisted of cat fur, cat urine, or approximately 0.1mL of pure essential lavender oil (Imperial Drug & Spice Corp., West New York, NJ) presented on Kimwipe. The cat fur odor cue was a piece of polyester Berber fleece that had been used as a bed for several different cats in the animal care facility for approximately 12 months. The fleece was cut into squares of approximately 5×7.5 cm and stored at -80 degrees Celsius. The cat urine stimulus was urine collected from a 16 year-old spayed female domestic cat. Urine was stored at -80 degrees Celsius and approximately 2 mL presented on a Kimwipe during exposure. Lavender oil was used as the control since pilot studies showed that rats do not display fear behaviors to this odorant.

2.4 Behavioral Measures

Two commonly studied kinds of fear responses were quantified: freezing and risk assessment. Freezing, the absence of all movement except for what is necessary for respiration, is a common fear response of adult rats encountering a cat or cat odor in the laboratory (Blanchard and Blanchard, 1971; Hubbard et al., 2004) and has been seen at younger ages (Wiedenmayer et al., 2005). Risk-assessment was quantified as the sum of stretch attend (head extended toward stimulus with low, flat back), flatback approach (slow movement towards stimulus with flat back), and head-up (head movement with nose in the air) behaviors (described by Dielenberg and McGregor, 2001; Hubbard et al., 2004). Behavior was recorded using a Panasonic PV-GS300 digital video camcorder mounted next to the chamber. Videos were transferred to a computer hard-drive then analyzed with The Observer XT 7.0 (Noldus Information Technology) and screened by an observer unaware of the experimental conditions. All behaviors were quantified for duration.

2.5 Procedure

To reduce handling-associated stress, the experimenter picked up and briefly (~120 sec) held each juvenile, adolescent, and adult animal on the 2 days prior to odor exposure to reduce handling-associated stress (Levine, 2005). At 11:00 A.M., under dim light conditions in the testing room, each rat was exposed to one of the three stimuli in either a small or large chamber. During exposure, a single animal was placed in the apparatus and allowed to habituate for two minutes. One stimulus condition was then presented via the vent port to the animal in the chamber for three minutes. The order of stimulus conditions, chamber size, and the sex of animals were counterbalanced. Animals were separated from littermates after exposure until all animals for the session had been run. In order to conserve body heat, infant animals were placed on a heating pad between sessions and prior to returning to the home cage.

2.6 Data Analysis

A three-way multivariate analysis of variance (MANOVA) was conducted to determine the effect of the three types of stimuli (cat fur, cat urine and control odor), the two sizes of chambers (small and large), and four ages (infant, juvenile, adolescent, and adult) on the two dependent variables, freezing and risk-assessment. Analyses of variances (ANOVAs) on the dependent variables were conducted as follow-up tests to the MANOVA. Using the Bonferroni method, each ANOVA was tested at the .025 level (.05 divided by the number of dependent variables) to control for Type I error. When a significant interaction was found, post hoc tests were conducted to evaluate the pairwise differences among the means with α set at .025. All statistical tests were performed using SPSS (v. 17 for Windows, Chicago, IL) and all graphs were generated using GraphPad Prism (v. 5 for Windows, San Diego, CA).

3. Results

3.1 Effects of Age

The 4 Age $\times$ 3 Stimulus $\times$ 2 Chamber Size MANOVA indicated a significant 4 $\times$ 3 interaction between age and the stimulus, Wilks's $\Lambda = 0.74$, $F_{(12, 238)} = 3.22$, $p < .001$. There was no effect of chamber size on the dependent variables of freezing (Figure 2) and risk-assessment (Figure 3), Wilks's $\Lambda = 0.98$, $F_{(2, 119)} = 1.24$, $p = .29$.

As predicted, differences were found in freezing behavior (ANOVA significant 4 $\times$ 3 interaction: $F_{(6, 120)} = 4.39$, $p < .001$) among the four ages in response to the three different stimuli (Figure 4). Contrary to our expectations, however, there were no statistical differences between ages on the dependent measure of risk-assessment ($F_{(6, 120)} = 2.32$, $p = .04$) to the various stimuli (Figure 5). Infants froze more to cat fur than did juveniles ($p < .01$), adolescents ($p < .001$), or adults ($p < .001$). Infants also displayed increased freezing behavior to cat urine than adolescents ($p < .001$) and adults ($p < .025$). Juveniles froze more to cat fur than adolescents ($p < .025$) and adults ($p < .01$). There were no differences in freezing between adolescents and adults. In sum, the youngest two groups of animals froze more to cat odor cues than the older two groups but the older animals did not engage in more risk-assessment than the younger animals.

3.2 Effects of the Stimulus

It was predicted that animals would display increased fear-related behavior to cat fur over all other stimuli. As reported above, this prediction was supported for the measure of freezing but not for the measure of risk-assessment. Although all ages of animals froze more during exposure to cat fur compared to the control odor (infants and juveniles: $p < .001$; adolescents and adults: $p < .025$), the youngest two ages additionally froze more during exposure to cat urine compared to the control odor (infants: $p < .001$; juveniles: $p < .025$) (Figure 6).

Surprisingly, adults were the only group that did not freeze more to cat fur than cat urine (infants: $p < .001$; juveniles: $p < .01$; adolescents: $p < .025$) (Figure 6).

4. Discussion

Unlearned fear behavior in rats changed as a function of age and the stimulus, but not of chamber size, partially supporting the hypothesis. Though all ages displayed fear-related behavior to cat odors, differences were reflected only in freezing behavior and not, as expected, risk-assessment. High levels of freezing behavior in infants and juveniles support the hypothesis that younger animals utilize this defensive strategy preferentially over risk-assessment.

One possible explanation for the lack of differences in risk-assessment behaviors in the older animals is that, in response to cat odor, the adult rat's natural defensive strategy is to hide and then engage in freezing and vigilant risk-assessment in order to decrease conspicuousness and attend to the threat (Blanchard and Blanchard, 1989; Dielenberg and McGregor, 2001). Without a place to hide, the rat may engage in a variety of other behaviors such as exploration as they seek out a hiding place. Sigmundi (1997) and de Oca et al. (2007) have gone so far to suggest that flight to an enclosure and freezing are both part of a "single defensive strategy". Indeed, the levels of freezing to cat fur in adults were lower than have been reported elsewhere (Li et al., 2004; Takahashi et al., 2007). The sum of the behaviors that make up the single strategy of risk-assessment may also have introduced variability, making it a less reliable a measure of unlearned fear-related behavior than freezing. It is also possible that sex differences on different measures of antipredator defensive behavior in the older animals dampened the effect (Shepherd et al., 1992). For example, adult female Long-Evans rats exposed to a cat-rubbed block increase the duration of stretch-attends and flatback approaches compared to males, but these differences do not reach statistical significance (Blanchard et al., 1990).

The mechanisms mediating unlearned fear responses to ecologically-relevant stimuli have likely been shaped by evolutionary processes, as adaptive behavioral responses are expected to be different across the lifespan when ecological threats change (Wiedenmayer, 2009), physical abilities develop, and home-ranges expand. For example, neonatal rats are naturally under less predation risk when sheltered in a burrow. On the other hand, when attacked, they may not have the physical coordination to flee. Both inexperience and physical immaturity could mean that heightened fear-related behavior (i.e. freezing) is both appropriate and adaptive behavior for such young animals. Around weaning, rats may naturally engage in more risk-assessment behavior if separated from the dam and their littermates. Peri-adolescent rats, who are hyperactive under naturalistic conditions, may display heightened exploratory and risk-assessment behaviors (Spear and Brake, 1983). The reduction of freezing to cat fur with increasing age appears to relate to the vulnerability of the animal at each stage of development.

That animals unanimously responded with fear to cat fur compared to the control odor was expected, based on findings with adult rats (for review, see Takahashi et al., 2008). However, the increased fear to cat urine than the control odor in infants and juveniles is a novel finding. This finding does not entirely conform to Blanchard et al.'s (2003b) suggestion that scatological cues are less threatening than cat fur, especially as adult animals did not respond to cat urine significantly less than cat fur. A likely explanation for high levels of freezing to cat urine in young animals is that these age groups possess limited defensive options to cope with threat and so may respond with freezing to all predator stimuli. For adults, this result was likely an artifact of low freezing to cat fur (Li et al., 2004; Takahashi et al., 2007).

The sizes of the chamber had no effect on either freezing or risk-assessment in this study. It may be that the chamber sizes used were not dissimilar enough to induce differences in unlearned fear-related behavior.

In sum, predator odors induced unconditioned fear across ontogeny and provided the opportunity to study the mechanisms underlying unlearned fear. These fear behaviors can become pathological when responsivity is exaggerated and anxiety becomes abnormal (Lima, 1998; Nesse, 1999; Ohman and Mineka, 2001). In these cases, long-term stimulation-induced changes model human psychiatric disorders (Rosen and Schulkin, 1998; LeDoux, 2000; Shekhar et al., 2001). Future studies should further address how natural stressors induce innate and learned fear across development.

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Figure 1. Odor conditioning apparatus

Rats are exposed to cat cues that are presented at the odor port. The size of the chamber is adjusted by a divider wall.

Figure 2. Freezing behavior is unaffected by chamber size
Mean and SEM percent time freezing during odor exposure ($N = 6$ per group).

Figure 3. Risk-assessment does not change with chamber size

Mean and SEM percent time engaging in risk-assessment behaviors during odor exposure ($N = 6$ per group).

Figure 4. Cat fur is a highly effective fear-inducing stimulus across the lifespan

Mean and SEM percent time freezing during odor exposure. For the effect of age, infants and juveniles displayed increased levels of freezing to cat odor cues compared to adolescents and adults. Because there was no effect of chamber size on freezing behavior, data for each stimulus condition are collapsed over this factor (** $p < .001$, ** $p < .01$, * $p < .025$; $N = 12$ per group).

Figure 5. Risk-assessment does not differ across age or stimulus

Mean and SEM percent time engaging in risk-assessment behaviors during odor exposure. Data for each stimulus condition are collapsed over chamber size ($N = 12$ per group).

Figure 6. Fear responsivity to different cat odor cues changes throughout life
Mean and SEM percent time freezing during odor exposure. Cat urine additionally increased freezing in younger animals compared to the control odor (***) $p < .001$, ** $p < .01$, * $p < .025$; $N = 12$ per group).