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Research report

Accessory and main olfactory systems influences on predator odor-induced behavioral and endocrine stress responses in rats

Cher V. Masini*, Robert J. Garcia, Sarah K. Sasse, Tara J. Nyhuis, Heidi E.W. Day, Serge Campeau

Department of Psychology and Neuroscience & Center for Neuroscience, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

Exposures to predator odors are very effective methods to evoke a variety of stress responses in rodents. We have previously found that ferret odor exposure leads to changes in endocrine hormones (corticosterone and ACTH) and behavior. To distinguish the contributions of the main and accessory olfactory systems in these responses, studies were designed to interfere with these two systems either independently, or simultaneously. Male Sprague–Dawley rats were treated with 10% zinc sulfate ($ZnSO_4$), which renders rodents anosmic (unable to smell) while leaving the accessory olfactory areas intact, or saline, in Experiment 1. In Experiment 2, the vomeronasal organs of rats were surgically removed (VNX) to block accessory olfactory processing, while leaving the main olfactory system intact. And in the third experiment both the main and accessory olfactory areas were disrupted by combining the two procedures in the same rats. Neither $ZnSO_4$ treatment nor VNX alone reliably reduced the increased corticosterone response to ferret odor compared to strawberry odor, but in combination, they did. This suggests that processing through the main *or* the accessory olfactory system can elicit the endocrine stress response to ferret odor. VNX alone also did not affect the behavioral responses to the ferret odor. $ZnSO_4$ treatment, alone and in combination with VNX, led to changes in behavior in response to both ferret and strawberry odor, making the behavioral results less clearly interpretable. Overall these studies suggest that both the main and accessory olfactory systems mediate the neuroendocrine response to predator odor.

1. Introduction

The presentation of predators or cues associated with them elicits increases in the stress hormones, corticosterone and adrenocorticotropin (ACTH), defensive behavioral responses, and autonomic nervous system activation [8,9,11–14,16,25,26,32,36]. Predators and their cues offer a unique model to study stress and anxiety, and this class of stimuli have been suggested as important models to study the development of mood disorders. Predator stress may model aspects of PTSD including changes in hypothalamic–pituitary–adrenal (HPA) axis function, widespread central nervous system effects, and long-term anxiety-like behavior [1–3]. There are several advantages for the use of predators and especially their associated cues for the study of stress and anxiety. Predator odor itself is not noxious and does not cause physical pain [7,10,32]. This is a significant advantage when studying the neural circuitry underlying stress/anxiety responses.

It has been suggested that predator odors are processed through a pheromonal, allomonal, or kairomonal pathway [6,13,31,39,48–50]; these odors activate the vomeronasal organ (VNO), which sends afferents to the accessory olfactory bulb (AOB) and ultimately project to the posteroventral, anterodorsal, and posterodorsal medial amygdala, among others, which are considered the 'vomeronasal amygdala' [18,19,23,33,37]. McGregor et al. found higher Fos protein immunoreactivity in the posterior AOB and posteroventral medial amygdala after cat odor compared to control odor exposure, but additionally reported higher Fos levels in the glomerular layer of the main olfactory bulb in the cat odor exposed compared to control odor exposed rats [31]. Likewise, higher c-*fos* mRNA expression was found in the posteroventral and posterodorsal medial amygdala after ferret odor exposure compared to a control odor exposure, but ferret odor exposed rats also had significantly higher levels of c-*fos* mRNA in several regions generally considered to be part of the main olfactory system, including the piriform cortex [25]. Traditionally, pheromones were thought to be processed exclusively by the accessory olfactory system, but recent studies have shown that the main olfactory system is also activated by known pheromonal odors [17,20,22,38,51], and the accessory olfactory system has been found to be reactive to non-pheromonal odors as well [41,44]. These recent findings lead to the conclusion that immediate-early gene expression alone may not provide the

* Corresponding author at: Department of Psychology and Neuroscience, University of Colorado, UCB 345, Boulder, CO 80309-0345, USA. Tel.: +1 303 735 2154; fax: +1 303 492 2967.
E-mail addresses: cher.masini@colorado.edu, masinic@colorado.edu (C.V. Masini).

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answer to the question of whether the main or accessory olfactory system independently or in combination mediate the effects of predator odors. The immunohistochemical and *in situ* hybridization techniques used only show that predator odors may be detected by these two olfactory systems. The techniques do not distinguish whether it is the accessory olfactory or main olfactory bulb activation that functionally induces the constellation of responses elicited by predator odors. A recent study in mice suggests that the dorsal (D1) domain of the main olfactory bulb mediates some of the effects of predator odors [20]; unfortunately, no functional data were presented with regard to putative activation, or lack thereof, of the accessory olfactory system with predator odor exposure in these specific olfactory knock-out mice.

The goal of the present study therefore, was to distinguish the contributions of the main and accessory olfactory systems in the defensive behavioral and neuroendocrine responses, as measured with plasma corticosterone levels, to ferret odor by blocking these two systems independently, or simultaneously. In our laboratory we have demonstrated that the fur/skin odor from a ferret is a highly potent stimulus that has long-lasting effects on behavior, HPA axis, and autonomic responses [9,25,26]. Zinc sulfate ($ZnSO_4$), which renders rodents anosmic while leaving the accessory olfactory areas intact, was used to temporarily disrupt main olfactory processing. Accessory olfactory processing was disrupted by surgically removing the vomeronasal organ. Defensive behavioral responses to a ferret odor stimulus were examined in a defensive withdrawal paradigm and plasma corticosterone response was examined in a home cage exposure paradigm. Surprisingly, neither manipulation alone reliably reduced the neuroendocrine and behavioral responses, suggesting that both systems can independently contribute to predator odor-induced responses. Some of these data have been presented in abstract form [27,28].

2. Methods

2.1. Experiment 1

2.1.1. Subjects

Forty-eight male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 200–250 g at the time of arrival to the colony were used. They were group-housed (4/cage) in a room kept on a controlled light–dark cycle (lights on 7:00 a.m. and off 7:00 p.m.) under constant humidity and temperature conditions. The rats were acclimated to the animal colony for a period of 7 days after arrival from the supplier, prior to any experimental manipulation. Rats were provided with food (rat chow) and water *ad libitum*. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1.2. Zinc sulfate treatment

One day before anosmia and behavioral testing, the rats were treated with zinc sulfate ($ZnSO_4$), to render them anosmic ($n = 22$), or saline ($n = 18$), as a control procedure. The rats were anesthetized with halothane and placed on their backs on an inclined surface with their heads facing downward. A curved 20-gauge syringe was put into the pharynx at the caudal end of the palate and slowly retracted rostrally to allow the tip to enter the nasal cavity via the posterior choanae. $ZnSO_4$ (10%, w/v in 0.9% saline; Ricca Chemical Co., Arlington, TX) was slowly perfused until a few drops were seen draining out of the external nares. The mouth of the rat was aspirated to remove saliva and excess solution during the procedure and during recovery from the anesthesia to prevent swallowing that could lead to sickness or death [5]. The sham-treated control rats were anesthetized and the nasal cavity perfused with saline instead of zinc sulfate. Post-treatment, all rats were individually housed in polycarbonate plastic cages (49.5 cm × 27.9 cm × 20.3 cm).

2.1.3. Anosmia testing

The next morning, to ensure that the rats were anosmic after $ZnSO_4$ treatment, a cookie-finding task was administered. An Oreo cookie with a diameter of 4.5 cm (Nabisco), which the rats were previously familiarized with during acclimation to the animal colony, was placed in a polycarbonate plastic cage (49.5 cm × 27.9 cm × 20.3 cm) and woodchip bedding poured on top of the cookie and spread in the cage to a depth of 3 cm. A rat was placed in the middle of the cage and a stopwatch started. If the $ZnSO_4$ treated rat did not actively find the cookie in 10 min, the rat was considered anosmic and included in the study. Previous studies have revealed that normal rats typically find the cookie within 2 min [24].

2.1.4. Odor stimuli

Ferret odor was collected by placing a small hand towel in a cage with breeding adult ferrets for approximately 1 month (courtesy Mile High Ferret Club). The towel was cut into 5 cm × 5 cm squares and kept in an −80 °C freezer until use. The towels were then thawed in a glass bell jar for 30 min before use. Strawberry odor was used as a novel control odor [25,26]. Towels were scented with strawberry odor by pipetting 100 μl of strawberry extract (McCormick & Co., Inc., Hunt Valley, MD) onto clean 5 cm × 5 cm towels.

2.1.5. Behavior testing

The rats were habituated to the defensive withdrawal apparatus by placing each rat in the open field for 10 min on three consecutive days at approximately the same time each day. The apparatus consisted of a 58 cm × 58 cm × 39 cm Plexiglas open field chamber with a metal 29 cm × 20 cm × 14 cm chamber in one corner with an opening on the long side that is 9 cm wide and 8 cm tall. The floor and sides of the open field were black and white tape was used to delineate 16 equal sized squares on the floor, of which the smaller chamber occupies two full squares and half of two squares. The room that contains the apparatus was dimly lit by a 75-W red light bulb and white noise (60 dB sound pressure level) was provided by an AM7 Grass Medical Instruments audio monitor (Quincy, MA).

The rats were tested the evening after $ZnSO_4$ or saline treatment after anosmia testing during the rats' dark phase. A piece of towel (ferret or strawberry odor) was taped to the floor of the open field diagonally opposite the withdrawal chamber. The rat was placed in the open field chamber, across from the withdrawal chamber and behavior was videotaped (Sony VHS recorder) for 10 min by a Panasonic WV-BP130 video camera (Ontario, Canada) that was mounted directly above the apparatus (approximately 2.4 m). An additional control group with naïve rats that had neither zinc sulfate nor saline treatment ($n = 8$) were placed in the defensive withdrawal paradigm without any towel stimulus to determine normal behavior without an odor influence.

Two researchers blind to the experimental conditions analyzed the videotaped behavior. Behaviors analyzed included: time spent in withdrawal chamber (s), time spent in the corner containing the towel (front paws in towel square), and number of rears (front paws leave floor). The scores of the two researchers were averaged. Scorers normally achieve a level of 95–99% agreement depending on the measure.

2.1.6. Home cage odor exposures

Immediately following behavior testing, the rats were placed back in their individual home cages, which were then placed in wood sound-attenuating chambers over night (kept on the same light cycle) to avoid manipulation and transport of the rats immediately prior to odor exposure. The next morning (rats' light/inactive phase), four pieces of towel (ferret or strawberry odor) were carefully placed in each corner of the cages without disturbing the rats by hooking the towels to the wire cage lid with paper clips, so the towels hung inside the cage approximately 5 cm from the floor. Rats were exposed to the opposite odor in their home cage as they had been exposed to in the defensive withdrawal paradigm, so that each rat received each odor once. Immediately following the 30 min towel exposure, the rats were taken to an adjacent room, decapitated, and trunk blood was collected.

2.1.7. Corticosterone ELISA

Blood was collected into ice-chilled tubes containing EDTA (20 mg/ml). Blood samples were then centrifuged at 2000 rpm for 10 min, the plasma pipetted into 0.5 ml Eppendorf microcentrifuge tubes, and stored at −80 °C until assayed. The corticosterone assay was performed according to the manufacturer's instructions (kit #901-097 – AssayDesigns, Ann Arbor, MI). Levels were then quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

2.1.8. Data analysis

Corticosterone data was analyzed using multivariate analyses of variance (MANOVA; Pillai's Trace) with treatment ($ZnSO_4$ or saline) and odor (ferret or strawberry) as the between-subjects factors. Behavioral data were analyzed using multivariate analyses of variance (MANOVA; Pillai's Trace) with treatment ($ZnSO_4$, saline, or no treatment) and odor (ferret, strawberry, or no odor) as the between-subjects factors. Post hoc comparisons were made using a Bonferroni test ($p < 0.05$).

2.2. Experiment 2

2.2.1. Subjects

Thirty-six male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 200–250 g at the time of arrival to the colony were used. Animals were housed as described in Experiment 1.

2.2.2. Vomeronasal organ removal surgery (VNX)

At least 1 week before testing, rats had their vomeronasal organs (VNO) surgically removed. The surgical procedure for the removal of the VNO was adapted from Wysocki and Wysocki (1995) [52]. The rats were anesthetized with an intramuscular injection of a solution of xylazine (60 mg/kg) and ketamine (21 mg/kg). The rats were then placed ventral side up, the lower jaw of the rat was retracted and an incision was made in the palate just caudal to the incisors to the second palatal ridge