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Discrimination of anxiety- versus panic-like behavior in the wall lizard *Tropidurus oreadicus*

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Abstract

A behavioral test battery is proposed for wall lizards (Tropidurus oreadicus) that consists of inducing tonic immobility (TI) followed by post-TI behavioral scoring. After the induction of TI, the usual behavioral sequence was flight followed by freezing and tongue-flicking and/or thigmotaxis, with flight being more probable than freezing. These sequences were not observed after restraint in a normal upward position (which induced freezing but not TI) or after handling (which increased the probability of tongue-flicking). Alprazolam and imipramine selectively decreased the duration of TI as well as the following flight and freezing behavior. Tongue-flicking was increased by diazepam and alprazolam, whereas fluoxetine decreased it. Finally, thigmotaxis was reduced by diazepam, alprazolam, and imipramine but increased by fluoxetine. These results suggest that panic and anxiety can be discriminated pharmacologically in wall lizards.
Keywords: wall lizard, panic, anxiety, defensive behavior, Tropidurus oreadicus.

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Introduction

Fear and anxiety are labels given to patterns of behavioral and neurovegetative adjustments that are associated with antipredator defense (Kavaliers & Choleris, 2001; McNaughton & Corr, 2004). In mammals, fear/panic and anxiety can be dissociated from an anatomical (McNaughton & Corr, 2004; McNaughton & Zangrossi, 2008; Panksepp, 2006) and pharmacological (Blanchard, Griebel, & Blanchard, 2001; Blanchard, Griebel, Henrie, & Blanchard, 1997) point of view. These effects demonstrate the pharmacological validity of such models because they mirror the sensitivity of generalized anxiety disorder (GAD) *vs*. panic disorder (PD) to different drugs. For example, GAD and its animal models are susceptible to treatment with the 5-hydroxytryptamine-1A receptor partial agonist buspirone, but this drug is ineffective in the treatment of PD. Conversely, monoamine oxidase inhibitors are effective in the treatment of PD but not GAD. Although benzodiazepines are clinically effective in the treatment of GAD, only the more potent triazolo-benzodiazepines are effective against PD. Chronic treatment with selective serotonin reuptake inhibitors is effective in the treatment of both disorders (Barlow, 2002).

The observation that basal vertebrates, such as zebrafish, also present defensive behavior that can be discriminated pharmacologically (Iturriaga-Vásquez, Osorio, Riquelme, Castro, & Herzog, 2012; Maximino et al., 2012; Maximino, da Silva, Gouveia, & Herculano, 2011; Stewart et al., 2011a,b; Subbiah & Kar, 2013) suggests that a separation between anxiety and fear is a primitive trait in vertebrates. Nonetheless, the hypothesis of homoplasy cannot be discarded without analyzing similar functions in intermediate taxa such as amphibians and reptiles. Many different types of defensive behavior have been described in small reptiles including tonic immobility (TI; Davies, Martinez-Garcia, Lanuza, & Novejarque, 2002; Hennig, 1979; dos Santos, de Oliveira, Verrastro, & Tozetti, 2010), flight (Hennig, 1979a), modifications in exploratory behavior (Cooper, 1994; Greenberg, 2002), and refuge use (López, Hawlena, Polo, Amo, & Martín, 2005).

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The advantages of using small reptiles as laboratory models were enumerated by Lovern, Holmes, and Wade (2004)and include their high availability in the wild (allowing for the establishment of laboratory colonies that are continuously replenished with wild stock to obtain field-relevant laboratory studies), the variety of life history traits among species, their close phylogenetic relationship with birds (forming the most basal extant amniotes), and ease of maintenance and cost-effectiveness without sacrificing ecological relevance.

Diverse manipulations have produced alterations in these behavioral endpoints in Sauria including lesions of the central nucleus of the amygdala (Davies et al., 2002) and the administration of stress-related hormones (Stratton & Kastin, 1976). Nonetheless, an attempt to systematize the defensive behavior of Sauria and its neuropharmacological underpinnings has not yet been made. Here we describe a simple manipulation (i.e., the induction of TI by inversion and restraint followed by the observation of exploratory behavior in an open field) that is amenable to treatment with diazepam, imipramine, fluoxetine, and alprazolam. We demonstrated that different behavioral endpoints were affected by different drug treatments, with remarkable correspondence to results observed in the rodent literature (e.g., the mouse defense test battery; Blanchard et al., 2001) and clinical efficacy data (Barlow, 2002).

Materials and methods

Animals and husbandry

Sixty adult lizards (*Tropidurus oreadicus*) of either sex, ranging from 80-118 mm in snout-cloaca length, were captured in Belém, PA, Brazil during February and March. The animals were inspected for mites, which were removed with forceps before treatment with de-miting solution as described by the manufacturer (Reptile Relief, Natural Chemistry, Norwalk, CT, USA). All of the lizards were treated with 50 mg/kg fenbendazole, p.o. and then housed according to recommendations for anoline lizards (Sanger, Hime, Johnson, Diani, & Losos, 2008) for at least 2 weeks before the experiments began. Briefly, the animals were housed in groups of four in standard rat cages (42 cm length x 27.5 cm width x 21 cm height) with mango tree sticks collected from the outdoors to provide perches. Before using the sticks, they were sterilized for 15 min in an autoclave. To prevent escape, screen meshes were inserted in the cage tops. The bottoms of the cages were covered with synthetic cage carpet (Repti Cage Carpet CC-10, Zoo Med, Costa Mesa, CA, USA) placed above a heater plate (Repti Therm U.T.H. Under Tank RH-6, Zoo Med) that kept the temperature above the carpet at an average of 28°C. The cages were misted with water twice daily, thus raising the humidity within each cage to approximately 85% (Sanger et al., 2008). The animals had *ad libitum* access to drinking water. The animals were fed three times weekly with commercial ration (Shrimp mix, Nutral, Monte Mor, Brazil) and once per week with captured crickets.

Experiment 1: Behavioral validation

Ten animals were used in Experiment 1. The animals were collectively transported to the experimental room 1 h before experiments began. Each animal was exposed to three sequences of TI, handling, and restraint in a counterbalanced fashion. Tonic immobility was induced by placing the animal on its back in the center of a 10 cm diameter circular open field and applying pressure to the thorax and pelvis while restraining the limbs. When the lizard ceased struggling, it was slowly released, and the time taken for it to resume an upright posture was recorded (Hennig, 1979). Handled control trials were conducted by equivalently handling the animals before introduction to the open field, without the induction of TI. Restrained animals were placed in their normal stance in the center of the open field, and their movement was restrained for 5 min. Each of these manipulations was made three times for each animal, and the trials were presented in a counterbalanced fashion.

The following behavioral endpoints were recorded after each of these manipulations: freezing (i.e., the lack of limb, neck, or tongue movements for more than 5 s in an upright position), flight (i.e., a high-velocity escape attempt with a latency of less than 10 s after release, usually leading to circling around the edges of the apparatus), and tongue-flicking (i.e., repeatedly licking the air with the tongue). These behavioral endpoints were manually recorded using EthoLog 2.2 (Ottoni, 2000), and the frequencies and duration were calculated. Behavioral sequence matrices were also extracted with EthoLog, and the *a posteriori* Bayesian probability of a given transition was extracted from those matrices using EthoSeq (Japyassú, Alberts, Izar, & Sato, 2006). The data were analyzed using two-way (manipulation *vs*. outcome) analyses of variance (ANOVAs) followed by Bonferroni tests whenever appropriate and are expressed graphically as mean ± SEM.

Experiment 2: Pharmacological analysis

Five groups of animals (n = 8 per group) were intraperitoneally injected with vehicle (0.5% dimethylsulfoxide [DMSO]), .1 mg/kg alprazolam, .5 mg/kg diazepam, 1 mg/kg imipramine, or 5 mg/kg fluoxetine before being subjected to the TI procedure. Only one TI trial was conducted, and the following behavioral variables were recorded: TI duration and latency, freezing duration, circling behavior frequency, tongue-flicking frequency, thigmotaxis (i.e., the ratio between time spent in the center and time spent in the periphery of the apparatus), and total locomotion (i.e., number of 1 cm^2 squares crossed). The data were analyzed using one-way ANOVAs, followed by Dunnett's test whenever appropriate.

Results

Experiment 1

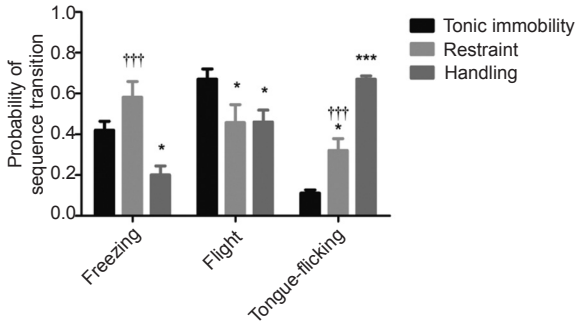

Figure 1. Conditional probability of freezing, flight, and tongue-flicking after the induction of tonic immobility, restraint, and handling stress. ***$p < .001$, compared with tonic immobility; *$p < .05$, compared with tonic immobility; †††$p < .001$, compared with handling.

No main effects of manipulation (induction of tonic immobility, restraint, or handling) were observed ($F_{2,81} = 0.77$, $p > .05$). Nonetheless, statistically significant differences were observed in terms of the conditional probability of specific outcomes (freezing, flight, and tongue-flicking) after each manipulation ($F_{2,81} = 7.718$, $p = .001$). Importantly, a significant interaction was observed ($F_{4,81} = 21.09$, $p < .0001$). Figure 1 presents the conditional probability results. Flight was more probable after TI than restraint, and tongue-flicking was more probable after restraint than TI ($p < 0.05$, Bonferroni *post hoc* test). Freezing and flight were more probable after TI than handling, and tongue-flicking was more probable after handling than TI ($p < .05$, Bonferroni *post hoc* test). Freezing was more probable after restraint than handling, and tongue-flicking was more probable after handling than restraint ($p < .05$, Bonferroni *post hoc* test).

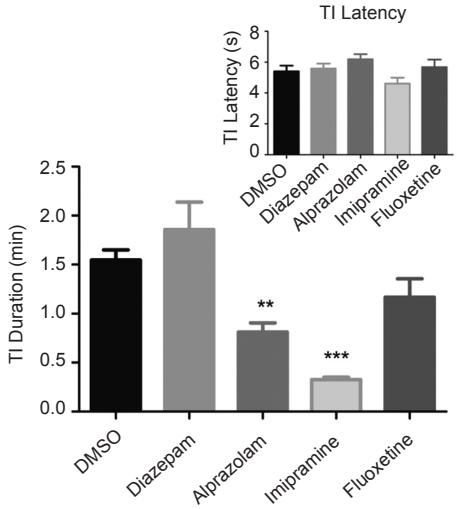

Figure 2. Effects of vehicle (DMSO), diazepam, alprazolam, imipramine, and fluoxetine on tonic immobility duration. ***$p < .001$, **$p < .01$, compared with DMSO. (Inset) No effect of these drugs was observed on TI latency.

Experiment 2

A main effect of drug treatment on TI duration was observed ($F_{4,39} = 13.52$, $p < .0001$; Figure 2), with alprazolam and imipramine decreasing TI compared with vehicle ($p < .01$, Dunnett's multiple comparison test). The latency to TI was not affected by any drug treatment in wall lizards ($F_{4,39} = 2.241$, $p > .05$), averaging 5.5 s (Figure 2, inset). As in Experiment 1, after reassuming the normal posture, the animals engaged in either explosive behavior (circling, freezing) or exploratory behavior (tongue-flicking, ambulation). Alprazolam and imipramine decreased freezing duration ($F_{4,39} = 5.875$, $p = .001$; Figure 3A) and the number of circling events ($H = 31.14$, $p < .0001$; Figure 3B). Both diazepam and alprazolam increased, whereas fluoxetine decreased, the frequency of tongue-flicking events ($H = 33.81$, $p < .0001$; Figure 4A). Thigmotaxis was reduced by diazepam, alprazolam, and imipramine and increased by fluoxetine ($F_{4,39} = 35.93$, $p < .0001$; Figure 4B). Overall locomotion was not affected by any drug treatment ($H = 7.089$, $p > .05$; Figure 4C).

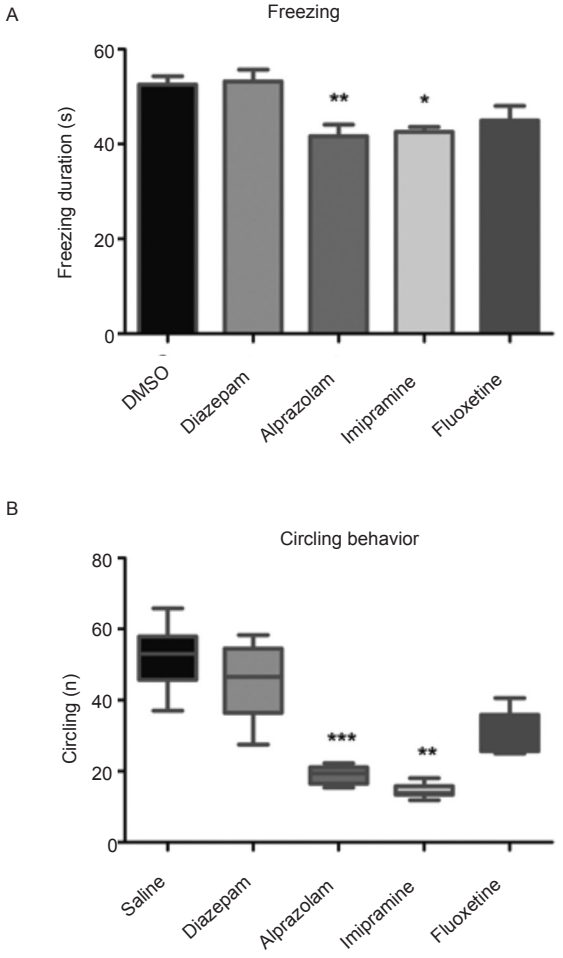

Figure 3. (A) Effects of vehicle (DMSO), diazepam, alprazolam, imipramine, and fluoxetine on freezing duration. (B) Effects of DMSO, diazepam, alprazolam, imipramine, and fluoxetine on the number of circling events. ***$p < .001$, **$p < .01$, *$p < .05$, compared with DMSO.

Discussion

The present study found that TI is affected by treatment with panicolytic but not anxiolytic drugs in the wall lizard *Tropidurus oreadicus*, demonstrating that this procedure can be used to induce a stereotypical sequence of defensive ("explosive") and exploratory behavior. The typical sequence (Experiment 1) was TI (approximately 1.5 min duration), followed by either circling or freezing for approximately 1 min. After this phase, a second phase of careful exploration (characterized by thigmotaxis and/or tongue-flicking) extended for the remainder of the 10-min session. Panicolytic drugs (alprazolam, imipramine) selectively decreased the duration of TI and the subsequent circling and freezing behavior. Normal exploratory behavior (tongue-flicking) was increased by anxiolytic drugs, whereas thigmotaxis was decreased.

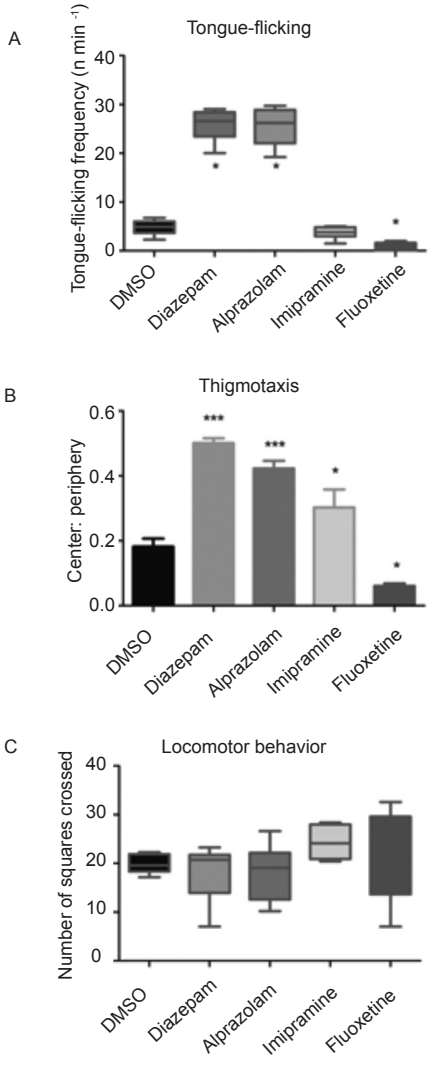

Figure 4. (A) Effects of vehicle (DMSO), diazepam, alprazolam, imipramine, and fluoxetine on the frequency of tongue-flicking events. (B) Effects of vehicle (DMSO), diazepam, alprazolam, imipramine, and fluoxetine on thigmotaxis (ratio of time spent in the center *vs*. periphery of the apparatus). (C) Effects of vehicle (DMSO), diazepam, alprazolam, imipramine, and fluoxetine on locomotion (number of squares crossed). ***$p < .001$, **$p < .01$, *$p < .05$, compared with DMSO.

Tonic immobility has been observed in species from all vertebrate taxa (Gallup, 1974) and is usually thought of as a "last resort" in a sequence of antipredator behavior (Ratner, 1967). In anoles, fear-inducing manipulations (e.g., the presence of predators or simulated predation) increase the duration of TI (Edson & Gallup, 1972; Hennig, Dunlap, & Gallup, 1976; Hennig, 1977, 1979). In *Podarcis hispanica*, lesions of the striato-amygdaloid transition area (considered homologous to the mammalian central nucleus of the amygdala) reduce the duration of TI (Davies et al., 2002). In rodents, however, the pharmacology of TI is less widely studied. In guinea pigs, benzodiazepine receptor agonists increase the duration of TI, whereas reverse agonists decrease it. Moreover, SSRIs have no effect (Olsen, Hogg, & Lapiz, 2002). These results are the exact opposite of what was observed in the present work. This discrepancy could be explained by the higher reliance on TI ("death-feigning") as a defense strategy in lizards, possibly because of metabolic constraints on activity bouts (Edson & Gallup, 1972; dos Santos et al., 2010).

Importantly, the present study observed behavior *after* the termination of immobility. Immediately after TI, the animals tended to exhibit more flight reactions than freezing reactions. Because of the shape and dimensions of the apparatus, flight took the form of "circling." Both freezing and circling were decreased by treatment with the triazolo-benzodiazepine alprazolam and tricyclic antidepressant imipramine, suggesting that they are alternative responses that are controlled by the same circuitry (i.e., a "fight/flight/freeze" system; Gray & McNaughton, 2000; McNaughton & Corr, 2004; McNaughton & Zangrossi, 2008) that organizes fear/panic-like behavior. Following flight/freeze reactions, cautious exploratory behavior ensues, with animals spending more time in the center than in the periphery of the open field (thigmotaxis) and performing tongue-flicking maneuvers, which are thought to be related to (vomeronasal) olfactory behavior (Cooper, 1994).

Overall, the present results suggest that *Tropidurus oreadicus* lizards have a well-organized pattern of defensive behavior that is amenable to pharmacological dissociation, and the behavioral effects of psychopharmacologicals are analogous (if not homologous) to observations in mammals. In addition to complementing our knowledge of the comparative psychopharmacology of anxiolysis, these results suggest the possible use of such animals in behavioral pharmacological research, an important development for researchers in underdeveloped areas of the world where the costs of maintaining a rodent vivarium are prohibitively expensive. Thus, the use of wall lizards as experimental subjects (a practice that is common in the field of neuroendocrinology; Crews and Moore, 2005) is a promising strategy in behavioral neuroscience.

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Competing interests statement

The authors declare no competing interests.

Author contributions

CM, CMC, and SM conceived and designed the experiments. CM and MGL performed the experiments. CM and SM analyzed the data. CM and SM wrote the paper.

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