

FGIN-1-27, an agonist at translocator protein 18 kDa (TSPO), produces anti-anxiety and anti-panic effects in non-mammalian models

Monica Gomes Lima¹, Jonathan Cueto-Escobedo², Juan Francisco Rodríguez-Landa², Caio Maximino³

¹ Departamento de Morfologia e Ciências Fisiológicas, Universidade do Estado do Pará – Campus VIII, Marabá/Brazil

² Laboratorio de Neurofarmacología, Instituto de Neuroetología, Universidad Veracruzana, Xalapa/Mexico

³ Instituto de Estudos em Saúde e Biológicas, Universidade Federal do Sul e Sudeste do Pará, Marabá/Brazil

Corresponding author

Caio Maximino

Instituto de Estudos em Saúde e Biológicas – Universidade Federal do Sul e Sudeste do Pará

Campus III – Av. dos Ipês, s/n

Marabá/PA, Brazil

cmaximino@unifesspa.edu.br

FGIN-1-27 is an agonist at the translocator protein 18 kDa (TSPO), a cholesterol transporter that is associated with neurosteroidogenesis. This protein has been identified as a peripheral binding site for benzodiazepines; in anamniotes, however, a second TSPO isoform that is absent in amniotes has been implicated in erythropoiesis. Functional conservation of the central benzodiazepine binding site located in the GABA_A receptors has been demonstrated in anamniotes and amniotes alike; however, the same was not previously demonstrated for TSPO. The present investigation explored the behavioral effects of FGIN-1-27 on an anxiety test in zebrafish (*Danio rerio*) and on a mixed anxiety/panic test on wall lizards (*Tropidurus oreadicus*). Results showed that FGIN-1-27 reduced anxiety-like behavior in the zebrafish light/dark preference test similar to diazepam, but with fewer sedative effects. Similarly, FGIN-1-27 also reduced anxiety- and fear-like behaviors in the defense test battery in wall lizards, again producing fewer sedative-like effects than diazepam; the benzodiazepine was also unable to reduce fear-like behaviors in this species. These results A) underline the functional conservation of TSPO in defensive behavior in anamniotes; B) strengthen the proposal of using anamniote behavior as models in behavioral pharmacology; and C) suggest TSPO/neurosteroidogenesis as a target in treating anxiety disorders.

Keywords: translocator protein 18 kDa; fear; anxiety; zebrafish; wall lizard; benzodiazepines

1. Introduction

The translocator protein 18 kDa (TSPO, mitochondrial benzodiazepine receptor, peripheral benzodiazepine receptor) was first identified as a peripheral binding site for diazepam, but later identified as part of the mitochondrial cholesterol transport pathway that is associated with the regulation of cellular proliferation, immunomodulation, porphyrin transport and heme biosynthesis, anion transport, regulation of steroidogenesis, and apoptosis (Casellas et al., 2002). This transporter is highly expressed in steroidogenic tissues. In the central nervous system, its expression is mainly restricted to ependymal cells and glia, in which it is responsible for the local synthesis of neuroactive steroids such as allopregnanolone (Papadopoulos et al., 2006). This latter neurosteroid, in its turn, positively modulates GABA_A receptors, especially those involved in tonic inhibition (Smith et al., 2009; Maguire et al., 2012), producing anxiolytic- (Fernández-Guasti and Picazo, 1995; Schüle et al., 2014) and antidepressant-like effects (Khisti et al., 2000; Rodríguez-Landa et

1 al., 2007, 2009), and modulates panic attacks (Bali and Jaggi, 2014; Lovick, 2014). As such, TSPO
2 has been proposed as a pharmacological target for the treatment of neurological and psychiatric
3 disorders associated with decreased GABAergic tone, such as anxiety disorders (Romeo et al.,
4 1993; de Mateos-Verchere et al., 1998; Kita et al., 2004; Costa et al., 2011; Matsuda et al., 2011;
5 Nin et al., 2011; Pinna and Rasmusson, 2012; Pinna and Rasmusson, 2012; Perna et al., 2014) and
6 epilepsy (Ugale et al., 2004), as well as for the fine control of stress responses (Gunn et al., 2011;
7 Maguire et al., 2012; Maguire, 2014). TSPO agonists produce anti-anxiety and anti-conflict effects
8 in rodents with both systemic (Kita et al., 2004; Costa et al., 2011) and intra-hippocampal (Bitran et
9 al. 2000) injections; these effects are blocked by GABA_A receptor antagonists (i.e. picrotoxin)
10 and/or 5 α -reductase blockers (i.e. 4-MA) and TSPO antagonists (PK 11195), implicating
11 neurosteroidogenesis and chloride ion channel at the GABA_A receptors in these responses (Bitran et
12 al. 2000). These effects are spared in adrenalectomized and castrated animals, suggesting that they
13 are not mediated by peripheral steroidogenesis, but rather by the production of neurosteroids in the
14 brain (Romeo et al., 1993). Nonetheless, octadecaneuropeptide, a diazepam-binding inhibitor
15 peptide which acts through both the central benzodiazepine receptor (CBR) and TSPO, produces
16 anxiety-like behavior in mice and rats (de Mateos-Verchere et al., 1998), as well as in goldfish
17 (Matsuda et al., 2011).

18 TSPO is highly conserved, being present in Bacteria, Archaea and Eukarya domains (Fan
19 and Papadopoulos, 2013). Anamniotes and invertebrates possess a single isoform, while amniotes
20 possess two TSPO isoforms (Fan et al., 2009). Interestingly, while no functional divergence is
21 predicted to appear between *tspo* (found in invertebrates and basal vertebrates) and *tspo1* (found in
22 amniotes), a functional divergence was detected in TSPO2 (Fan and Papadopoulos, 2013). Some of
23 the neurobehavioral functions of this protein, on the other hand, seen to be conserved. In zebrafish,
24 for example, benzodiazepines have been shown to affect a plethora of anxiety-like behaviors, from
25 bottom-dwelling (Bencan et al., 2009; Egan et al., 2009) and dark preference (Maximino et al.,

2010, 2011) to shoal cohesion (Gebauer et al., 2011) and cocaine withdrawal-induced anxiety (López-Patiño et al., 2008). Likewise, benzodiazepines decrease tonic immobility duration and the following freezing and explosive behavior in a defensive behavior battery in the wall lizard (*Tropidurus oreadicus*), and also increase exploratory behavior in the same test (Maximino et al., 2014). In the separation stress paradigm, benzodiazepines attenuate separation stress-induced distress vocalizations in chicks in the anxiety phase, but not in the depression phase (Warnick et al., 2009). Thus, agonists at the CBR decrease fear- and anxiety-like behavior in both amniotes and anamniotes. Moreover, some evidence regarding the neurosteroidogenesis pathway in behavioral control has been suggested by the observation that allopregnanolone has an anticonvulsant effect in zebrafish (Baxendale et al. 2012), and that chronic fluoxetine treatment upregulates the expression of genes from the neurosteroidogenesis pathway in this species (Wong et al. 2013). These results suggest that some downstream effectors of neurosteroidogenesis are conserved, although it is not known whether the role of TSPO in behavioral control *per se* is conserved. A comparative approach could untangle this question, especially if species at the base of the amniote and anamniote clades are used. In this paper, we describe the behavioral effects of FGIN-1-27, a TSPO agonist, in zebrafish (*Danio rerio* Hamilton 1822, Cyprinidae) and wall lizards (*Tropidurus oreadicus* Rodrigues, Tropiduridae) and compare these responses with the effects of diazepam, an agonist at the CBR with tested anxiolytic and anticonvulsant actions at preclinical and clinical research.

19

20 **2. Experimental procedures**

21 **2.1. Experiment 1: Effects of FGIN-1-27 and diazepam on dark preference in zebrafish**

22 *2.1.1. Animals and husbandry*

23 120 adult zebrafish from the *longfin* phenotype were acquired in a local aquarium shop and kept in
24 collective tanks at the laboratory for at least 2 weeks before experiments started. Conditions in the
25 maintenance tank were kept stable, as described by Lawrence (2007). Recommendations in the

1 Brazilian legislation (Conselho Nacional de Controle de Experimentação Animal - CONCEA,
2 2017) were followed to ensure ethical principles in animal care and throughout experiments. This
3 manuscript is a complete report of all the studies performed to test the effect of diazepam or FGIN-
4 1-27 on anxiety-like behavior in zebrafish. We report how the sample size was determined , all data
5 exclusions (if any), all manipulations, and all measures in the study.

6 7 2.1.2. Drug administration

8 Diazepam was dissolved in 40 % propylene glycol, 10 % ethyl alcohol, 5 % sodium benzoate, and
9 1.5 % benzyl alcohol (Maximino et al. 2010). FGIN-1-27 was dissolved in 1% DMSO to which one
10 or two drops of Tween 80 was added before sonication into a fine suspension (Auta et al. 1993).
11 Drugs were diluted to their final concentrations and injected *i.p.* in a volume of 1 μ L/0.1 g b.w.
12 (Kinkel et al. 2010). For both diazepam and FGIN-1-27, the doses used were 0.14, 0.28, 0.57, 1.1,
13 and 2.3 mg/kg . The three highest doses were chosen based on reported effects of diazepam on
14 zebrafish (Maximino et al. 2010) or wall lizard (Maximino et al. 2014) defensive behavior, and
15 were identical for both drugs to facilitate comparison; the lower doses were chosen in order to
16 define a minimal effect. All drugs were acquired from Tocris Bioscience (UK).

17 18 2.1.3. Experimental groups and dosage

19 The adult zebrafish were assigned to 12 independent groups (n= 10 fish per group): a vehicle group
20 (propylene glycol:ethyl alcohol:sodium benzoate:benzyl alcohol for the diazepam controls, DSMO
21 for the FGIN-1-27 controls) that received 1 μ L/0.1 g b.w.; and 10 additional groups that received
22 the doses described above for diazepam (5 groups) or FGIN-1-27 (5 groups). Sample sizes were
23 based on Maximino *et al.* (2010). All drugs were *i.p.* injected in a volume of 1 μ L/0.1 g b.w., 30
24 min before of the behavioral test. Animals were randomly drawn from the holding tank immediately
25 before injection, and the order with which doses were tested was randomized *via* generation of ran-
26 dom numbers using the randomization tool in <http://www.randomization.com/>. Experimenters were

1 blinded to treatment by using coded vials for drugs. The data analyst was blinded to phenotype by
2 using coding to reflect treatments in the resulting datasets; after analysis, data was unblinded.

3

4 2.1.4. Scototaxis assay

5 The light/dark preference (scototaxis) assay was performed as described by Maximino *et al.*,
6 (2013). Briefly, 30 min after injection animals were transferred individually to the central
7 compartment of a black and white tank (15 cm height X 10 cm width X 45 cm length) for a 3-min
8 acclimation period, after which the doors which delimit this compartment were removed and the an-
9 imal was allowed to freely explore the apparatus for 15 min. While the whole experimental tank
10 was illuminated from above by an homogeneous light source, due to the reflectivity of the apparatus
11 walls and floor average illumination (measured just above the water line) above the black compart-
12 ment was 225 ± 64.2 (mean \pm S.D.) lux, while in the white compartment it was 307 ± 96.7 lux. The
13 following variables were recorded:

14 *time spent on the white compartment*: the time spent in the white half of the tank (percentage of
15 the trial);

16 *squares crossed*: the number of 10 cm² squares crossed by the animal in the white compart-
17 ment;

18 *latency to white compartment*: the time in seconds (s) to first entry in the white compartment;

19 *erratic swimming*: defined as the number of zig-zag, fast, and unpredictable swimming behav-
20 ior of short duration;

21 *freezing*: the proportional duration of freezing events (in % of time in the white compartment),
22 defined as complete cessation of movements with the exception of eye and operculum move-
23 ments;

24 *thigmotaxis*: the proportional duration of thigmotaxis events (in % of time in the white com-
25 partment), defined as swimming in a distance of 2 cm or less from the white compartment's

walls;

risk assessment: the number of “risk assessment” events, defined as a fast (<1 s) entry in the white compartment followed by re-entry in the black compartment, or as a partial entry in the white compartment (i.e., the pectoral fin does not cross the midline);

A digital video camera (Samsung ES68, Carl Zeiss lens) was installed above the apparatus to record the behavioral activity of the zebrafish. Two independent observers, blinded to treatment, manually measured the behavioral variables using X-Plo-Rat 2005 (<https://github.com/lanec-unifesspa/x-plo-rat>). Inter-observer reliability was at least > 0.85.

2.1.5. Statistical analysis

The data were analyzed using two-way ANOVAs, with drug and dose as between-subjects factors. Values of $p \leq 0.05$ in the ANOVA were followed by Tukey's HSD whenever appropriate; planned comparisons were between different doses of a given drug and its vehicle and between same doses of both drugs. All statistical analyses were made using R 3.1.3. Data are presented graphically as means \pm S.E.M.

2.1.6. Data availability

Full data can be found in our GitHub repository (<https://github.com/lanec-unifesspa/fgin-1-27/tree/master/zebrafish>)

2.2. Experiment 2: Effects of FGIN-1-27 and diazepam on the defense test battery in *Tropidurus oreadicus*

2.2.1. Animals and husbandry

120 adult wall lizards (*Tropidurus oreadicus*) of either sex, ranging from 61-96 mm in rostro-cloacal length, were captured in Marabá/PA, Brazil, between February and March 2015. The animals were inspected for mites, which were removed with forceps before treatment with de-miting solu-

tion as described by the manufacturer (Reptile Relief, Natural Chemistry, Norwalk, USA). All of the lizards were treated with 50 mg/kg fenbendazole (Vetnil, Brazil), p.o., and then housed according to recommendations for anoline lizards (Sanger et al. 2008) for at least 2 weeks before the experiments began. Animals were housed in groups of four in Plexiglas standard laboratory 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, USA) placed above a heater plate (Repti Therm U.T.H. Under Tank RH-6, Zoo Med, Costa Mesa, USA) 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, Mexico) and once per week with captured crickets. In the absence of Brazilian guidelines for lizards, the guidelines for the husbandry of *Anolis* lizards by Sanger *et al.* (2008) were used, in an attempt to ensure ethical principles in animal care throughout experiments.

2.2.2. Drug administration

FGIN-1-27 and diazepam were prepared as in Experiment 1 and injected intraperitoneally in a volume of 0.5 ml of either vehicle or drug 30 min before behavioral tests.

2.2.3. Experimental groups and dosage

Adult lizards were assigned to 12 independent groups (n= 10 lizards per group): a vehicle group (propylene glycol:ethyl alcohol:sodium benzoate:benzyl alcohol for the diazepam controls, DMSO for the FGIN-1-27 controls) that received 1 µL/0.1 g b.w.; and 10 additional groups that received

the doses described above for diazepam (5 groups) or FGIN-1-27 (5 groups). Sample sizes were based on Maximino *et al.* (2014). All drugs were *i.p.* injected in a volume of 0.5 ml per animal, 30 min before of the behavioral test. Animals were randomly drawn from the cage immediately before injection, and the order with which doses were tested was randomized *via* generation of random numbers using the randomization tool in <http://www.randomization.com/>. Experimenters were blinded to treatment by using coded vials for drugs. The data analyst was blinded to phenotype by using coding to reflect treatments in the resulting datasets; after analysis, data was unblinded.

2.2.4. Defense test battery

The defense test battery was applied as described by Maximino *et al.* (2014). Briefly, tonic immobility was induced by carefully placing the animal on its back in the center of a 10 cm diameter circular open field and applying mild 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). After the animal spontaneously ceased tonic immobility (TI), the following behavioral endpoints were recorded after each of these manipulations:

tonic immobility (TI): the total duration, in minutes (min), in a rigid supine posture after release;

freezing: the lack of limb, neck, or tongue movements for more than 5 s in an upright position;

circling: 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 quantified as the number of complete circles made near the walls;

tongue-flicking: repeatedly licking the air with the tongue;

ventilatory frequency: the average number of inspiratory responses per minute;

total locomotion: the number of 2 cm² squares crossed by normal locomotor responses (i.e.,

not concomitant to circling); can be superimposed to tongue-flicking.

A digital video camera (Samsung ES68, Carl Zeiss lens) was installed above the apparatus to record the behavioral activity of the lizards. Two independent observers, blinded to treatment, manually measured the behavioral variables using EthoLog 2.2 (Ottoni 2000). Inter-observer reliability was at least > 0.85 .

2.2.5 Statistical analysis

The data were analyzed using two-way ANOVAs, with drug and dose as between-subjects factors. Values of $p \leq 0.05$ in the ANOVA were followed by Tukey's HSD whenever appropriate; planned comparisons were between different doses of a given drug and its vehicle and between same doses of both drugs. All statistical analyses were made using R 3.1.3. Data are presented graphically as means \pm S.E.M.

2.2.6. Data availability

Full data can be found in our GitHub repository (<https://github.com/lanec-unifesspa/fgin-1-27/tree/master/lizards>)

3. Results

3.1. Experiment 1

Main effects of drug ($F_{1,107} = 91.216$, $p < 0.0001$) and dose ($F_{5,107} = 32.173$, $p < 0.0001$), as well as an interaction effect ($F_{5,107} = 4.939$, $p = 0.000416$) were observed in time spent into the white compartment (Figure 1A). Post-hoc tests uncovered differences between all doses except the highest in relation to controls in diazepam-treated animals ($p < 0.001$) and between all doses in relation to controls in FGIN-1-27-treated animals ($p < 0.001$). Finally, differences were also observed between FGIN-1-27- and diazepam-treated animals at doses of 0.56 mg/kg ($p = 0.0012$), 1.2 mg/kg ($p < 0.0001$), and 2.4 mg/kg ($p = 0.0002$).

1 Main effects of drug ($F_{1, 107} = 17.64$, $p < 0.0001$) and dose ($F_{5, 107} = 207.77$, $p < 0.0001$), as well as an
2 interaction effect ($F_{5, 107} = 20.11$, $p < 0.0001$), were found for risk assessment (Figure 1B). Post-hoc
3 tests uncovered differences between vehicle-treated and diazepam-treated animals at all doses ($p <$
4 0.0001), and between vehicle-treated and FGIN-1-27-treated animals at all doses ($p < 0.0001$).
5 Moreover, differences between diazepam- and FGIN-1-27-treated animals were observed at 1.1
6 mg/kg ($p = 0.012$) and 2.3 mg/kg ($p < 0.0001$).
7 A main effect of dose ($F_{5, 107} = 111.547$, $p < 0.0001$), but not drug ($F_{1, 107} = 0.643$, $p = 0.424$), was
8 found for thigmotaxis (Figure 1C), and an interaction effect was also found ($F_{5, 107} = 10.536$, $p <$
9 0.0001). Post-hoc tests unveiled differences between vehicle-treated and diazepam-treated animals
10 at doses above 0.28 mg/kg ($p < 0.01$), as well as between all FGIN-1-27 doses and vehicle-treated
11 animals ($p < 0.0001$). Differences were also observed between diazepam- and FGIN-1-27-treated
12 zebrafish at doses of 0.14 mg/kg ($p = 0.0065$) and 1.1 mg/kg ($p = 0.044$).
13 Main effects of drug ($F_{1, 107} = 89.134$, $p < 0.0001$) and dose ($F_{5, 107} = 47.418$, $p < 0.0001$), as well as
14 an interaction effect ($F_{5, 107} = 6.921$, $p < 0.0001$), were found for freezing (Figure 1D). Post-hoc tests
15 uncovered differences between vehicle-treated and diazepam-treated animals at all doses ($p <$
16 0.001), and between FGIN-1-27-treated and vehicle-treated animals at 0.28 mg/kg and higher doses
17 ($p < 0.001$). Differences were also found between diazepam- and FGIN-1-27-treated zebrafish at
18 doses of 0.14 mg/kg ($p = 0.03$), 1.1 mg/kg ($p < 0.0001$), and 2.3 mg/kg ($p < 0.0001$).
19 Main effects of drug ($F_{1, 107} = 14.57$, $p = 0.000226$) and dose ($F_{5, 107} = 42.64$, $p < 0.0001$), as well as
20 an interaction effect ($F_{5, 107} = 26.48$, $p < 0.0001$), were found for erratic swimming (Figure 1E).
21 Post-tests uncovered no statistically significant differences between vehicle-treated and diazepam-
22 treated animals at any dose ($p > 0.05$), while FGIN-1-27-treated animals were significantly different
23 between vehicle-treated animals at doses of 0.28 mg/kg ($p = 0.0001$), 1.1 mg/kg ($p < 0.001$) and 2.3
24 mg/kg ($p < 0.0001$). Also, differences were observed between FGIN-1-27- and diazepam-treated
25 animals at 0.28 mg/kg ($p = 0.021$), 1.1 mg/kg ($p = 0.05$), and 2.3 mg/kg ($p < 0.0001$).

1 Finally, no main effects of drug ($F_{1, 107} = 3.526$, $p = 0.0631$), but a main dose effect ($F_{5, 107} = 9.088$, p
2 < 0.0001) as well as a suggestive interaction effect ($F_{5, 107} = 2.778$, $p = 0.0212$) were found for num-
3 ber of entries into the white compartment (Figure 1F). Post-hoc tests uncovered differences between
4 vehicle- and diazepam-treated zebrafish at doses of 0.28 mg/kg and 0.57 mg/kg ($p < 0.01$) and vehi-
5 cle- and FGIN-1-27-treated zebrafish at the highest dose ($p = 0.003$). No differences were found be-
6 tween diazepam- and FGIN-1-27-treated animals.

7 8 **3.2. Experiment 2**

9 Main effects of drug ($F_{1, 108} = 248.5$, $p < 0.0001$) and dose ($F_{1, 108} = 93.39$, $p < 0.0001$), as well as an
10 interaction effect ($F_{1, 108} = 60.09$, $p < 0.0001$) were found for TI duration (Figure 2A). Post-hoc tests
11 found statistically significant differences between vehicle- and diazepam-treated lizards at doses of
12 0.28-1.1 mg/kg ($p < 0.05$) and vehicle- and FGIN-1-27-treated lizards at all doses except the higher
13 ($p < 0.0001$). Differences between FGIN-1-27- and diazepam-treated lizards were observed at all
14 doses except the higher ($p < 0.0001$).

15 Main effects of drug ($F_{1, 108} = 771.29$, $p < 0.0001$) and dose ($F_{5, 108} = 59.58$, $p < 0.0001$), as well as an
16 interaction effect ($F_{5, 108} = 66.98$, $p < 0.0001$) were found for circling behavior (Figure 2B). Post-hoc
17 tests revealed differences between vehicle- and FGIN-1-27 treated lizards at all doses ($p < 0.0001$),
18 and between vehicle- and diazepam-treated animals at 1.1 and 2.3 mg/kg ($p < 0.05$).

19 Main effects of drug ($F_{1, 108} = 291.58$, $p < 0.0001$) and dose ($F_{5, 108} = 45.09$, $p < 0.0001$), as well as an
20 interaction effect ($F_{5, 108} = 14.63$, $p < 0.0001$) were found for freezing (Figure 2C). Post-hoc tests
21 identified significant differences between vehicle- and diazepam-treated animals at 1.1 mg/kg ($p <$
22 0.0001) and between vehicle- and FGIN-1-27-treated animals at all doses ($p < 0.0001$). Differences
23 between diazepam- and FGIN-1-27-treated lizards were also found for all doses ($p < 0.0001$).

24 A drug main effect ($F_{1, 108} = 1487.5$, $p < 0.0001$) and a dose main effect ($F_{5, 108} = 110.54$, $p <$
25 0.0001), as well as an interaction effect ($F_{5, 108} = 86.71$, $p < 0.0001$) were observed for ventilatory

frequency (Figure 2D). Post-hoc tests uncovered differences between vehicle- and diazepam-treated animals at 1.1 mg/kg ($p < 0.0001$), and between vehicle- and FGIN-1-27-treated animals at all doses ($p < 0.0001$). Finally, statistically significant differences were found between diazepam- and FGIN-1-27-treated animals at all doses ($p < 0.0001$).

Main effects of drug ($F_{1,108} = 34.26$, $p < 0.0001$) and dose ($F_{5,108} = 413.3$, $p < 0.0001$), as well as interaction effect ($F_{5,108} = 351.08$, $p < 0.0001$) were found for tongue-flicking (Figure 2E). Post-hoc tests revealed significant differences between vehicle- and diazepam-treated lizards at doses of 1.1 and 2.3 mg/kg ($p < 0.0001$), and between vehicle- and FGIN-1-27-treated lizards at all doses except the highest ($p < 0.0001$). Moreover, statistically significant differences between diazepam- and FGIN-1-27-treated lizards at all evaluated doses were found ($p < 0.0001$).

Main effects of both drug ($F_{1,108} = 91.53$, $p < 0.0001$) and dose ($F_{5,108} = 36.28$, $p < 0.0001$), as well as an interaction effect ($F_{5,108} = 56.62$, $p < 0.0001$) were found for total locomotion (Figure 2F). Significant differences were found between vehicle- and diazepam-treated lizards at 0.57 and 2.3 mg/kg ($p < 0.0003$) and vehicle- and FGIN-1-27-treated lizards at 1.1 and 2.3 mg/kg ($p < 0.002$). Finally, statistically significant differences were also found between diazepam- and FGIN-1-27-treated animals at 2.3 mg/kg ($p < 0.0001$).

4. Discussion

In the present work we demonstrated that the TSPO agonist FGIN-1-27 produced a dose-dependent decrease in defensive behavior in both wall lizards and zebrafish. Specifically, in wall lizards FGIN-1-27 decreased tonic immobility in the intermediate dose range (0.14-1.1 mg/kg); decreased post-TI ventilatory frequency, freezing and circling at all doses; and increased exploratory behavior (tongue-flicking) and decreased thigmotaxis at 0.14-1.1 mg/kg. Similarly, in zebrafish FGIN-1-27 decreased scototaxis, thigmotaxis, freezing and risk assessment at all doses, increasing erratic swimming at 0.14 and 0.28 mg/kg. The highest dose (2.3 mg/kg) decreased locomotion in both

1 species, suggesting a sedative effect. Moreover, diazepam, a CBR and TSPO agonist, decreased TI
2 duration at the smaller doses and increased it at high doses, increased tongue-flicking and decreased
3 thigmotaxis at doses above 0.28 mg/kg; no effects were observed in post-TI behavior. In zebrafish,
4 diazepam produced an inverted-U shape effect on scototaxis and entries into the white compart-
5 ment, monotonically decreasing freezing, thigmotaxis and risk assessment.

6 In the lizard defense test battery, induction of TI produces a stereotypical behavioral pattern in
7 which is followed by either freezing or “explosive” circling behavior; after that, the animal emits
8 exploratory behavior, marked by thigmotaxis (“wall-hugging”) and tongue-flicking (Maximino *et*
9 *al.*, 2014). This sequence resembles defensive behavior at increasing predatory imminence continua
10 (Fanselow and Lester, 1988) – that is, defensive behavior in this test shifts from panic-like, circa-
11 strike behavior towards sustained risk assessment (i.e., tongue-flicking, thigmotaxis). Moreover, in
12 a previous experiment panicolytic drugs (i.e., alprazolam, imipramine) decreased TI duration, freez-
13 ing and circling behavior, while diazepam (0.5 mg/kg) increased tongue-flicking and decreased
14 thigmotaxis (Maximino *et al.*, 2014). In the present experiment, FGIN-1-27 produced a more wide
15 range of effects than diazepam: the TSPO agonist affected exploratory behavior (consistent with an
16 anxiolytic-like effect), post-TI behavior (consistent with a panicolytic effect) and TI duration, which
17 could also represent a panicolytic-like effect. Diazepam, on the other hand, only affected ex-
18 ploratory behavior and had a hormetic (inverted-U shape) profile on TI duration.

19 A complementary profile was observed in the zebrafish scototaxis assay. Benzodiazepines have
20 been shown to produce a hormetic profile in several behavioral tests in zebrafish (Bencan *et al.*,
21 2009; Cachat *et al.*, 2010; Sackerman *et al.*, 2010; Vada *et al.*, 2015). Previous experiments also
22 demonstrated that diazepam was anxiolytic at 1.25 mg/kg, but not 2.5 mg/kg, in the scototaxis assay
23 (Maximino *et al.*, 2011); in addition to this observation, the present work also demonstrated effects
24 of diazepam on freezing, thigmotaxis and risk assessment. Importantly, FGIN-1-27 treatment also
25 produced anxiolytic-like effects, with locomotor-impairing effects at the highest dose.

1 Little is known about the role of TSPO in behavioral control in anamniotes. In goldfish (*Carassius*
2 *auratus* Linn, Cyprinidae), octadecaneuropeptide increased the latency to enter a white compart-
3 ment in a light/dark box, suggesting an anxiogenic-like effect (Matsuda et al., 2011). While octade-
4 caneuropeptide is an endozepine which acts as an agonist at TSPO (Papadopoulos et al., 1991), it
5 also acts as an antagonist at the CBR (Ferrero et al., 1986); consistently with the hypothesis that the
6 anxiogenic-like effect of octadecaneuropeptide is mediated by the CBR, flumazenil, but not a
7 metabotropic endozepine receptor antagonist, blocked the scototaxis effects in the goldfish (Mat-
8 suda et al. 2011).

9 Overall, the present results suggest that drugs targeting the CBR and MBR exert anti-anxiety effects
10 in anamniotes, while drugs acting at the MBR also exert anti-panic effects. It is plausible that some
11 of the effects of diazepam were mediated by the MBR; further experiments are needed to untangle
12 the precise mechanisms. Thus, while the present results suggest a functional conservation of TSPO
13 that is concomitant to gene duplication, it is not known whether FGIN-1-27 (or even diazepam) pro-
14 duced its behavioral effects in lizards and zebrafish by acting on the (conserved) *tspol* or whether
15 some effects are also mediated by *tspo*. Further experiments will clarify the issue. The present re-
16 sults also add to the mounting evidence that TSPO/MBR ligands could be used to treat fear disor-
17 ders, including panic disorder, in human populations. The degree of conservation of *tspol* suggest
18 that anamniotes could be used as experimental models to study potential anti-panic drugs to contrib-
19 ute to develop of pharmacological therapeutic strategies to ameliorate panic disorders symptoms in
20 the human.

21

22 **Acknowledgments**

23 This manuscript first appeared as a preprint on bioRxiv (<https://doi.org/10.1101/056507>).

24

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29

30 **Figure captions**

31 **Figure 1** – Effects of FGIN-1-27 (filled circles) and diazepam (empty circles) on (A) scototaxis, (B)
32 risk assessment, (C) thigmotaxis, (D) freezing, (E) erratic swimming, and (F) total locomotion in
33 the light/dark test in zebrafish (*Danio rerio*). Error bars represent the standard errors.

34

1 **Figure 2** – Effects of FGIN-1-27 (filled circles) and diazepam (empty circles) on (A) tonic immo-
2 bility, (B) circling responses, (C) freezing, (D) ventilatory frequency, (E) tongue-flicking, and (F)
3 total locomotion on wall lizards (*Tropidurus oreadicus*). Error bars represent the standard errors.



