The anxiolitic effects of BTG1640 and BTG1675A on ultrasonic isolation calls and locomotor activity of rat pups

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Abstract. - OBJECTIVE: The aim of the present study was to evaluate the anxiolytic properties of the new isoxazoline compounds BTG1640 and BTG1675A in comparison with diazepam.

MATERIALS AND METHODS: We evaluated the ultrasonic distress emission in both sexes of neonatal rat pups (which seems to be a sensitive indicator of the rat emotional reactivity and represents a valuable tool to screen compounds with expected anxiolytic properties) and the locomotor activity in 30-day old rat pups.

RESULTS: We found a significant reduction in the number of emitted ultrasonic calls only after i.p. administration of diazepam 1 mg/kg, while no significant reduction have been detected after i.p. administration of BTG 1640 and BTG 1675A. Furthermore, we found a significant reduction of locomotor activity in the first 10' of the test, only in the group treated with diazepam 0.1 mg.

CONCLUSIONS: The tests validating the supposed anxiolytic properties of the new isoxazoline compounds BTG1640 and BTG1675A, in comparison with diazepam, gave negative results.

Key Words: Ultrasonic vocalization, Isolation calls, Rat pups, Benzodiazepine receptors, Distress vocalization, Locomotor activity, Anxiolytics, Buspirone, Diazepam.

Introduction

Benzodiazepines are the most commonly prescribed anxiolytic drugs, being efficacious against a spectrum of anxiety disorders. However, there are issues with addiction, tolerance, and dependence/withdrawal, as well as adverse side effects that include sedation, cognitive and psychomotor impairment and anterograde amnesia. The other major classes of compounds mainly used to treat anxiety are selective serotonin reuptake inhibitors (SSRIs) and the 5HT-1A partial agonist, buspirone. However, both classes of compounds have a slow onset of action (4-6 weeks) and their own side profiles. There is, therefore, a need for anxiolytics showing a rapid onset of action and an efficacy similar to benzodiazepines, with a low abuse potential and minimal impairment of cognition and motor skills. Developmentally, the earliest known manifestation of an anxiety-like state is the response of rodent pups to isolation

When isolated for a brief period of time in the first 14 days of life, rat and mouse pups respond with species-specific ultrasonic distress or isolation calls that are potent stimuli for maternal retrieval. The benzodiazepine, serotonin and other biogenic amine receptor systems and endogenous opiate, CCK, CRF and other peptide systems, have shown to regulate the rate of calling in rat and mouse pup. Rat pups usually emit ultrasonic calls during brief episodes of social separation. These calls have been variously described as “distress” calls and may be related to the separation cries expressed by the young of many mammalian species. Ultrasonic call of rat pups is modulated by environmental stimuli such as ambient temperature, olfactory and tactile stimuli associated with the nest. Calls are also sensitive to a variety of purported anxiolytic and anxiogenic drugs, including the benzodiazepines, serotonin agonists, and ligands at the NMDA-glycine receptor complex. Experimental evidence also indicates the implication of the nitric oxide (NO) in anxiety. In 2015, Kalouda and Pitsikas investigated also the effects of the NO
Rat distress behavior elicited by anxiolytics

Donor molsidomine on anxiety-like behavior and compared them in rats with the anxiolytic diazepam using the light/dark and the open field tests. The effects of molsidomine on rat motility were also assessed. Molsidomine, at any dose tested, did not alter locomotor activity compared with vehicle-treated rats in a motility test. The results indicate that the 2 mg/kg molsidomine induced anxiolytic-like effects in the rat, during the light/dark and open field tests, cannot be attributed to changes in locomotor activity and that the magnitude of the molsidomine (2 mg/kg)-induced anxiolytic-like effects was not different to that produced by the benzodiazepine anxiolytic diazepam (1 mg/kg). In addition, to provide new insights about the development and neurobiology of anxiety, tests used in this work are well pharmacologically validated animal models to study the anxiolytic properties of drugs.

Materials and Methods

Animals and Treatment Schedule

Experiments were performed in accordance with guidelines released by Italian Ministry of Health (D.L. 116/92) and (D.L. 111/94-B), the Declaration of Helsinki and the “Guide for the Care and Use of Laboratory Animals” as adopted and promulgated by the National Institutes of Health.

Primiparous Wistar female rats (Harlan SRC, Milan, Italy) weighing 250-280 g were used. The animals were housed for two weeks before the experiment at constant room temperature (20-22°C) and humidity (60%), with a light cycle of 12 h/day (08.00 h-20.00 h) and food and water available ad libitum. Pairs of females were placed with single male rats in the late afternoon. Vaginal smears were taken the following morning at 09.00 h. The day on which sperms were present was designated as day 0 of gestation (GD 0). Pregnant rats were then randomly assigned to the nine experimental groups. At birth, each litter was reduced to a standard size of eight pups per litter (4 males and 4 females, when possible) within 24 h after birth. Pups were weaned at 21 days of age. After pharmacological administration, a total of 54 litters of Wistar rats were used and, among them, 27 litters were used to evaluate the ultrasonic response elicited by distress isolation and the other 27 litters were used to evaluate their motor activity.

Therefore, the experimental groups in this study were the following: one male and one female pup per litter from different litters per treatment group were used. Each couple of pups (1 male and 1 female) from each litter from different experimental groups were used only for a single test and tested once. Recording sessions were conducted, for each experimental group, immediately before and 20 min and 40 min after the pharmacological administration. For each dose, for each compound and for each sex, each experimental group (6-9 animals) was composed taking, in random mode, one pup from different litters.

Ultrasonic Vocalization in Rat Pups

Recording sessions were conducted in a sound-attenuating chamber (3.00 x 2.00 x 2.25 m), according to the technique previously described (1). Vocalizations of rat pups were recorded on a Racal Store 4DS high-speed tape-recorder using a direct mode recording procedure with a tape speed of 30 i.p.s. (76.8 cm/s); the frequency response range was flat between 200 Hz and 150 kHz. Ampex magnetic tapes (length: 3,280 feet, width: 0.25 inch) with precision reels were used. The transducers employed were a calibrated Bruel & Kjaer (mod. 4135) ¼ in (0.64 cm) free-field condenser microphone (frequency response flat within ± 3 dB, from 5 Hz to 100 kHz), a Bruel & Kjaer microphone preamplifier (mod. 2618) with a linear frequency response from 10 Hz to 200 kHz which provided 20 dB amplification, a Rank Precision Ind. low noise amplifier which provided 20 dB amplification steps and a Khron-Hite tunable band-pass filter (mod. 3500) set at 20 to 100 kHz. Acoustic characteristics of the signals were later analyzed on a Bruel & Kjaer (mod. 2033) High-Resolution Signal Analyzer (HRSA). This required a reduction of tape speed from the original 30 i.p.s. (inch/sec) (76.8 cm/s) to 3 ¾ i.p.s. (9.6 cm/s). Following this analysis, the expanded time base and the reduced frequency values were adjusted back to the original recording speed to estimate duration and frequency of the signal. Amplifying gains were taken into account to estimate sound pressure levels which were expressed in decibels re 2 x 10^-5 N/m^2 RMS (Root Mean Square). The rate of vocalization (no. of calls/15 s) was counted manually by listening to the audible output of the tape-recorder with loud-speaker and by watching the vocalization displayed on the HRSA on time-intensity mode during tape replay at 3 ¾ i.p.s. The duration of calls was obtained by measuring the HRSA six ultrasonic signals for each pup (one call every 20 s; the measure-
ment started 20 s after the beginning of the tape replay at 3 ¾ i.p.s.), from the HRSA the maximum and minimum frequency (kHz) as well as the peak pressure in decibels (re 2 x 10⁻⁵ N/m² RMS) of all the calls emitted by each pup during the 15 s recording session were measured during tape replay at 3 ¾ i.p.s. One male and one female pup from each litter were randomly removed from the nest. Thereafter, each pup was placed in a shallow plastic dish (15 cm in diameter and 6 cm deep) 15 sec before the test. This confined the pup movement relative to the microphone which was supported vertically 15 cm above the dish and thus avoided handling them during the recording session which lasted 15 sec. Each pup was tested at the 10th day of age (Table I).

**Locomotor Activity**

Locomotor activity was recorded in Makrolon cages by infrared monitoring, according to the technique previously described¹² and modified as follows. Briefly, a passive infrared sensor (RK 2000 QPC) was placed 23 cm above the centre of each cage (59 x 38.5 x 20 cm), which was covered with stainless steel wire lids. The infrared sensors were connected, via an interface to a PC, which was programmed to check all sensors at 1-s intervals. A sensor, which was turned on, i.e. registering no movement, gave a score of 0 for this interval, and a sensor, which was turned off, i.e. registering a movement, and gave a score of 1. These points were added and registered over 15-min blocks. Therefore, the theoretical range of activity for 5 min was 0-300. All tests were carried out in a 3 x 2 x 2 mt sound-attenuating cabin illuminated by a 20-W white light. A fan produced background noise of 46 dB. The animals were subjected to an hour session at 30 days of age. The test started when an animal was placed in the centre of the arena. Immediately after each test the apparatus was thoroughly cleaned by cotton pads wetted with 48% ethanol solution. In this experiment, one of the four male or female pups in each litter was randomly allocated to each of the nine experimental groups. Experimental groups consisted of 30 day old rats: controls; diazepam 0.1, diazepam 1 mg/kg; BTG 1640 0.1, 1 and 10 mg/kg; BTG 1675A 0.1, 1 and 10 mg/kg. Recording sessions were conducted, for each experimental group, immediately before and 20 min and 40 min after the pharmacological administration. For each dose, for each compound and for sex, each experimental group (6-9 rats) was composed taking, in random mode, one pup from different litters.

**Statistical Analysis**

Experimental groups were statistically evaluated by the “Analysis of Variance”. Individual comparisons were analyzed by the “Tukey’s test”. 

$p < 0.05$ was considered statistically significant.

**Results**

**Ultrasonic Vocalization in Rat Pups**

To evaluate the ultrasonic calls emitted by rat pups, we performed statistical tests at 20’ and 40’ after i.p. administration of the different substances (diazepam, BTG 1640 and BTG 1675A) at the following doses:

A Two-Way Analysis of Variance for repeated measures at a dose of 0.1 mg/kg, produced the following results:

- Treatments: $F = 0.49; \text{df} = 3/22, \text{n.s}$;
- Times: $F = 0.01; \text{df} = 2/44; \text{n.s}$;
- Interactions: $F = 1.28; \text{df} = 6/44; \text{n.s}$.

**Table I.** Groups of rats subjected to the tests of ultrasonic vocalization (10 days old) and locomotor activity (30 days old).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No of male rats</th>
<th>No of female rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Diazepam</td>
<td>0.1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Diazepam</td>
<td>1</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>0</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>BTG 1640</td>
<td>0.1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>BTG 1640</td>
<td>1</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>BTG 1640</td>
<td>10</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>Saline</td>
<td>0</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>BTG 1675A</td>
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<td>7</td>
</tr>
<tr>
<td>10</td>
<td>BTG 1675A</td>
<td>1</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>BTG 1675A</td>
<td>10</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>12</td>
<td>Saline</td>
<td>0</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
A Two-Way Analysis of Variance for repeated measures, at 20' and 40' after i.p. administration of the different substances at a dose of 1 mg/kg, produced the following results:

- **Treatments**: F = 10.38; df = 3/30; p < 0.001;
- **Times**: F = 7.56; df = 2/60; p < 0.002;
- **Interactions**: F = 4.78; df = 6/60; p < 0.001.

A Two-Way Analysis of Variance for repeated measures at 20' and 40' after i.p. administration of the different substances at a dose of 10 mg/kg, produced the following results:

- **Treatments**: F = 1.34; df = 2/19; n.s.
- **Times**: F = 2.40; df = 2/38; n.s.
- **Interactions**: F = 0.20; df = 4/38; n.s.

Individual comparisons of the emitted ultrasonic vocalizations resulted statistically significant only in the experimental group treated with diazepam:

- p < 0.01 vs. baseline (20' from administration)
- p < 0.05 vs. baseline (40' from administration)
- p < 0.05 vs. vehicle (20' e 40' from administration)

### Locomotor Activity

The Two-Way Analysis of Variance for repeated measures (value detected every 10' for a 40') at a dose of 0.1 mg/kg of diazepam, BTG 1640 and BTG 1675A, produced the following results:

- **Treatments**: F = 1.96; df = 3/24; n.s.
- **Times**: F = 26.1; df = 3/72; p < 0.001
- **Interactions**: F = 2.43; df = 9/72; p < 0.025

The Two-Way Analysis of Variance for repeated measures (value detected every 10' for a 40') test at a dose of 1 mg/kg of diazepam, BTG 1640 and BTG 1675A, produced the following results:

- **Treatments**: F = 3.80; df = 3/24; p < 0.025
- **Times**: F = 3.93; df = 3/72; p < 0.02
- **Interactions**: F = 1.56; df = 9/72; n.s.

Individual comparisons evidenced a significant difference, in the first 10' of the test, only in the group treated with diazepam on the control group (p < 0.01).

The Two-Way Analysis of Variance for repeated measures (value detected every 10' for a 40') test at a dose of 10 mg/kg of diazepam, BTG 1640 and BTG 1675A, produced the following results:

- **Treatments**: F = 0.73; df = 2/18; n.s.
- **Times**: F = 3.48; df = 3/54; p < 0.025
- **Interactions**: F = 0.80; df = 6/54; n.s.

### Discussion

Understanding the types and functions of ultrasonic vocalizations emitted by laborato-ry rodents may enable researchers and animal care personnel to use vocalizations as an indicator of an animal’s behavior and affect. Our results regarded mainly the manifestation of an anxiety-like state, like the response of rodent pups to isolation in novel environments. When isolated for a brief period of time in the first 14 days of life, rat and mouse pups respond with species-specific ultrasonic distress or isolation calls that are potent stimuli for maternal retrieval. In this research, we have found a significant reduction in the number of emitted ultrasonic calls after i.p. administration of diazepam 1 mg/kg, while no significant reduction have been detected after i.p. administration of BTG 1640 and BTG 1675A. Furthermore, a significant reduction of locomotor activity was found, in the first 10' of the test, only in the group treated with diazepam 0.1 mg. These calls, described as “distress” calls, may be related to the separation cries expressed by the young of many mammalian species. The results obtained in this research shed more light on the role of benzodiazepines on rat pup ultrasonic vocalization providing new insights about the development and neurobiology of anxiety, also because the performed tests are pharmacologically well-validated animal model to study the anxiolytic properties of drugs.

### Conclusions

Tests validating the supposed anxiolytic properties of the new isoxazoline compounds BTG1640 and BTG1675A in comparison with diazepam gave negative results.

### Conflicts of interest

The authors declare no conflicts of interest.

### References


