Behavioral Profile of L-655,708, a Selective Ligand for the Benzodiazepine Site of GABA-A Receptors Which Contain the α5 Subunit, in Social Encounters Between Male Mice

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GABA-A receptor is a transmembrane hetero-oligomeric protein which consists of five subunits, the combination of which confers unique pharmacological properties to the receptor. It is well-known that the GABAergic system is involved in the modulation of aggression. However, the role of α5/GABA-A receptors has not been explored. In this study, we examined the effect of L-655,708 (0.625-5 mg/kg), a selective ligand for the benzodiazepine site of GABA-A receptors which contain the α5 subunit, on agonistic behavior elicited by isolation in male mice. Individually housed mice were exposed to an anosmic “standard opponent” 30 min after drug administration, and the encounters were videotaped and evaluated using an ethologically based analysis. L-655,708 (5 mg/kg) exhibited an ethopharmacological profile characterized by a marked reduction of the time spent in offensive behavior (threat and attack) without affecting immobility, accompanied by a significant increase of avoidance/flee and nonsocial exploration behaviors, suggesting that the antiaggressive effect of the drug is unselective. Overall, this behavioral profile might indicate the existence of an anxiogenic-like activity of L-655,708 in mice. Aggr. Behav. 30:319–325, 2004. © 2004 Wiley-Liss, Inc.

Keywords: aggression; social encounters; α5/GABA-A receptors; L-655,708; anxiety; mice

INTRODUCTION

GABA-A receptors belong to the superfamily of ligand-gated ion channels mediating the bulk of fast inhibitory neurotransmission in the mammalian brain. They are a family of pentameric Cl- ion channels composed of subunits (α1-6, β1-4, γ1-4, δ, ρ1-3) that are encoded by genes with diverse expression patterns [Sieghart, 2000; Whiting, 1999]. Thus, the GABA-A receptors are formed by a wide diversity of combinations of subunits, whose expression varies in different brain regions and whose pharmacological properties also differ, creating an opportunity to target receptor subtypes with novel drugs [Kneusel et al., 2002; Korpi et al., 2002].

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It is well-known that GABAergic system is involved in the modulation of aggression [Miczek et al., 1994, 2002]. Thus, in animal studies, numerous ligands for GABA-A receptors appear to exhibit antiaggressive activity. Benzodiazepines (BDZ) such as diazepam [Martín-López and Navarro, 1997], clobazam [Martín-López and Navarro, 1996], bentazepam [Martín-López and Navarro, 1998], and midazolam [Martín-López and Navarro, 1999], as well as cyclopyrrolones and imidazopyridines such as zopiclone and zolpidem, respectively [Martín-López et al., 1994; Martín-López and Navarro, 2002; Ueki, 1987], have been demonstrated to possess antiaggressive properties in isolation-induced aggression models. Betacarbolines, which act as inverse agonists at GABA-A receptors, also may reduce aggressive behavior of isolated mice [Beltrán and Navarro, 2002]. This antiaggressive activity seems to be reversed by flumazenil administration [Miczek et al., 1994], an antagonist of GABA-A/BDZ receptors. Overall, these data underlie a significant role for the GABA-A receptors in modulating aggressive behavior.

Pharmacological analysis of GABA-A receptors immunoprecipitated with antibodies against specific α-subunit variants allows differentiating between GABA-A receptor subtypes. The α1, α2, α3 and α5 GABA-A receptors correspond to diazepam-sensitive receptors, whereas the α4- and α6-GABA-A receptors are insensitive to diazepam. The former are distinguished further by their affinity to zolpidem (α1 > α2 = α3), a drug that displays a very high affinity for the GABA-A containing α1 subunits, whereas it is insensitive to α5/GABA-A receptors [Wingrove et al., 2002]. Although the role of GABA-A receptors containing an α5 subunit has been scarcely examined, recent studies have suggested a possible implication of α5-GABA-A receptors in spatial learning [Collinson et al., 2002; Crestani et al., 2002] and anxiety [Navarro et al., 2002]. L-655,708 is a new, potent ligand selective for the benzodiazepine site of GABA-A receptor containing an α5 subunit [Quirk et al., 1996]. It is a partial inverse agonist that exhibits 100-fold higher affinity for α5 containing receptors, compared with α1 containing receptors [Casula et al., 2001]. Although α5/GABA-A receptors are mainly expressed in hippocampus [Li et al., 2001; Pirker et al., 2000; Sur et al., 1999], they are also present in other brain structures such as amygdala and hypothalamus [Fritschy and Brüning, 2003], which are involved in the modulation of aggression [Halasz et al., 2002; Lonstein and Gammie, 2002]. Therefore, we designed this experiment to explore the effects of a wide dose range of L-655,708 (0.625–5 mg/kg, ip) on agonistic encounters in isolated male mice using an ethopharmacological approach.

MATERIALS AND METHODS

Animals

A total of 184 albino male mice of the OF.1 strain (provided by CRIFFA, Barcelona, Spain) weighing 25–30 g were used. Animals were housed under standardized lighting conditions (white lights on: 20:00–8:00), at a constant temperature (21 °C) with food and tap water available ad libitum, except during behavioral trials. Upon arrival in the laboratory, mice were allocated to two different categories. Half were housed individually in transparent plastic cages (24 × 13.5 × 13 cm) as experimental animals and the remainder housed in groups of five to be used as “standard opponents” and were rendered temporally anosmic by intranasal lavage with 4% zinc sulphate solution (Sigma Laboratories) on both days 1 and 3 before testing. We used this type of opponent because it elicits attack but never initiates such
behavior [Brain et al., 1981]. These animals rarely direct spontaneous attacks towards the test animals and, consequently, fighting is always unidirectional, being thus easily quantified.

All experimental animals were kept in isolation for 30 days prior to behavioral testing (isolation-induced aggression model) since social isolation is an effective form of increasing the level of aggressiveness in different species of animals, particularly in laboratory mice [Navarro, 1997; Valzelli, 1969].

This experiment was carried out in accordance with the guiding principles for care and use of Laboratory Animals approved by the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Drug Administration

Six groups of mice were used. Animals were randomly allocated to two control groups (n = 16 each) receiving physiological saline (0.9% sodium chloride) or physiological saline plus dimethylsulphoxide (DMSO) and four experimental groups (n = 15 each) receiving L-655,708 injections. L-655,708 (Tocris Laboratories) was diluted in physiological saline (80%) plus DMSO (20%) to provide appropriate doses for injections and administered acutely in four doses: 0.625, 1.25, 2.5 and 5 mg/kg. Drug or vehicle was injected intraperitoneally in a volume of 10 ml/kg. Tests were performed 30 min after injections. The doses were chosen on the basis of a previous experiment carried out in our laboratory with this substance [Navarro et al., 2002].

Castro et al. [1995] have characterized the behavioral effects of different vehicles (including DMSO) commonly used in pharmacological experiments. Their results showed that DMSO only affected the behavior of animals at very high concentrations.

Agonistic Encounters and Behavioural Analysis

Thirty minutes after injection, an isolated animal and a “standard opponent” were allowed to confront each other in a neutral area for 10 min. This neutral cage consisted of an all glass area, measuring 50 x 26 x 30 cm with a fresh sawdust substrate. Before the encounter, the animals were allowed 1 min of adaptation to the neutral cage, remaining separated by a plastic barrier throughout this time. The social encounters were videotaped using a Sony-V8 camera. All tests were conducted under red light between the second and seventh hours of the dark phase of the artificial cycle of the animals. Individual animals were tested in a random order throughout this period. After each encounter, the neutral cage was washed out and the sawdust bedding was replaced. The tapes were analyzed using a microprocessor and a custom-developed programme [Brain et al., 1989], which facilitated estimation of time and frequency allocated to ten broad behavioral categories. The names of the categories and their constituent elements are as follows: (i) body care (which includes groom, self-groom, wash, shake, scratch); (ii) digging (dig, kick dig, push dig); (iii) non-social exploration (explore, rear, supported rear, scan); (iv) exploration from a distance (approach, attend, circle, head orient, stretched attention); (v) social investigation (crawl over, crawl under, follow, groom, head groom, investigate, nose sniff, sniff, push past, walk around); (vi) threat (aggressive groom, sideways offensive, upright offensive, tail rattle); (vii) attack (charge, lunge, attack, chase); (viii) avoidance/flee (evade, flinch, retreat, ricochet, wheel, startle, jump, leave, wall, clutch); (ix) defense/submission (upright defensive, upright submissive, sideways defensive), and (x) immobility (squat, cringe). This ethoexperimental procedure allows a complete quantification of the behavioral elements shown by the subject during the agonistic
encounters. Only the behavior of the isolated animal was assessed. The analysis was carried out by a trained experimenter who was unaware of the treatment administered to the groups.

**Statistical Analysis**

The medians for times allocated to each behavioral category were determined. Non-parametric Kruskal-Wallis tests were used to assess the variance of the behavioral measures over different treatment groups. Subsequently, appropriate paired comparisons were carried out using Mann-Whitney U-tests to contrast the behavior in the different treatment groups. The analysis was performed using non-parametric statistics since the criteria for the parametric statistics were not met by the data. The criterion for statistical significance for all the tests was \( P < .05 \).

**RESULTS**

The effects of acute administration of L-655,708 on agonistic interactions between male mice are shown in Table I (medians with ranges). Kruskal-Wallis analysis showed that this compound had significant effects on non-social exploration, threat, attack and avoidance/flee behaviors \( (P < .01) \). Post-hoc Mann-Whitney U-tests revealed that L-655,708 (5 mg/kg)

<table>
<thead>
<tr>
<th>Behavioral categories</th>
<th>Saline (min–max)</th>
<th>Saline + DMSO (min–max)</th>
<th>0.625 (min–max)</th>
<th>1.25 (min–max)</th>
<th>2.5 (min–max)</th>
<th>5 (min–max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body care</td>
<td>11.5 (0.7–43)</td>
<td>10.5 (2–47)</td>
<td>11.1 (6–41)</td>
<td>9.9 (2–24)</td>
<td>11.7 (4–35)</td>
<td>11.3 (3–58)</td>
</tr>
<tr>
<td>Digging</td>
<td>9.04 (0–27)</td>
<td>7.03 (0.56)</td>
<td>7.9 (1–21)</td>
<td>17.5 (0–71)</td>
<td>9.4 (0.5–47)</td>
<td>7.1 (0–24)</td>
</tr>
<tr>
<td>Nonsocial exploration( a )</td>
<td>329 (160–390)</td>
<td>349 (227–422)</td>
<td>337 (266–396)</td>
<td>371 (237–467)</td>
<td>354 (17–85)</td>
<td>432*</td>
</tr>
<tr>
<td>Exploration from a distance</td>
<td>52 (18–76)</td>
<td>55.6 (41–84)</td>
<td>56.9 (37–92)</td>
<td>54.9 (21–97)</td>
<td>45.9 (17–85)</td>
<td>64.3 (28–99)</td>
</tr>
<tr>
<td>Social investigation</td>
<td>37.2 (0–137)</td>
<td>39.7 (4–84)</td>
<td>56.5 (0–108)</td>
<td>50.1 (4–105)</td>
<td>40.1 (9–110)</td>
<td>67.6 (11–144)</td>
</tr>
<tr>
<td>Threat( a )</td>
<td>112 (40–293)</td>
<td>91.2 (21–131)</td>
<td>102.2 (0–149)</td>
<td>62.6 (4–138)</td>
<td>80.5 (24–141)</td>
<td>1.93**</td>
</tr>
<tr>
<td>Attack( a )</td>
<td>41.2 (0–79)</td>
<td>33.5 (0–86)</td>
<td>29 (0–110)</td>
<td>26 (0–83)</td>
<td>39 (5–67)</td>
<td>0**</td>
</tr>
<tr>
<td>Avoidance/flee( a )</td>
<td>0 (0–3.2)</td>
<td>0 (0–3.7)</td>
<td>0 (0–8)</td>
<td>0 (0–13)</td>
<td>0 (0–2)</td>
<td>2.57**</td>
</tr>
<tr>
<td>Defense/Submission</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0.8)</td>
<td>0 (0–0)</td>
<td>0 (0–0.5)</td>
<td>0 (0–0.5)</td>
</tr>
<tr>
<td>Immobility</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
</tbody>
</table>

Kruskal-Wallis test showed significant variance: \(^{a}P < .01\).

Differs from control groups on Mann-Whitney U-tests: \(^{a}P < .05; \ ^{**}P < .01\).
significantly reduced the time spent in threat and attack behaviors, as compared with the control groups (P < .01). Likewise, non-social exploration and avoidance/flee behaviors were significantly increased after treatment with the highest dose of the drug, in comparison with the control groups (P < .05 and P < .01, respectively). There were no significant differences between both control groups in any of the behavioral categories analyzed. The median values for defence/submission and immobility were zero for all groups.

DISCUSSION

The term agonistic behavior encompasses threats and aggressive acts as well as defensive, submissive and flight behaviors. The ethological analyses of these social encounters seems to be an appropriate technique to distinguish between specific and non-specific drug-induced changes. The results obtained in the present study indicate that L-655,708 exhibits an antiaggressive activity in isolated male mice. As Table I shows, acute treatment with L-655,708 (5 mg/kg) provoked a marked reduction of offensive behaviors (threat and attack), without a concomitant increase of immobility. However, other behavioral categories were clearly affected, suggesting that the antiaggressive action of L-655,708 was unselective. Thus, avoidance/flee and nonsocial exploration behaviors were significantly enhanced after treatment with the drug. Both behavioral categories have been traditionally used to assess the anxiety-changing properties of drugs [Brain et al., 1991]. In fact, increases of avoidance/flee behaviors are considered as good predictors of anxiogenic-like activity of drugs, whereas the increase of time spent in non-social exploration behaviors may reflect attempts to escape from the test arena. Therefore, overall, this ethopharmacological profile might indicate the existence of an anxiogenic-like activity of L-655,708 in mice. These findings are in concordance with a recent study in which an anxiogenic-like profile was described in mice using the elevated plus-maze test [Navarro et al., 2002]. The possible anxiogenic-like effect found in our study could be mediated by the α5-GABA-A receptors located over the hippocampal formation [Li et al., 2001; Sur et al., 1999], a region which has shown to be involved in the modulation of anxiety [DeGroot and Treit, 2003].

To our knowledge, this is the first report in which the behavioral profile of a selective ligand for the benzodiazepine site of GABA-A receptors containing the α5 subunit has been examined in agonistic encounters between male mice. Although the lack of experimental studies with these ligands does not permit us to compare directly our results with other works, there are published studies with several compounds which act at the GABA-A receptors containing other subunits. Thus, zolpidem has been shown to interact selectively with GABA-A receptors containing the α1 subunit. Zolpidem (1.5 mg/kg), in contrast to L-655,708, seems to exhibit a selective antiaggressive action, reducing significantly the offensive behaviours without affecting avoidance/flee and other behavioral categories related to anxiety [Martin-López and Navarro, 2002].

L-655,708 is a partial inverse agonist at the GABA-A receptors. Overall, our results are in agreement with those described with some betacarbolines (eg., FG 7142), which also act as partial inverse agonists (although without a selective affinity for any α subunit). These compounds have also demonstrated to reduce aggressive behavior of laboratory animals (and increase avoidance/flee behaviors) in different experimental models of aggression, such as muricide aggression [Nagatani et al., 1990], maternal aggression [Barseggian et al., 1987; Mos et al., 1987], social conflict [Beck and Cooper, 1986; Sulcová et al., 1992], and isolation-induced aggression [Beltrán and Navarro, 2002].
In sum, in this study we have shown that L-655,708, a selective ligand for the benzodiazepine site of GABA-A receptors which contain the α5 subunit, exhibits an antiaggressive action in agonistic encounters between male mice. However, this antiaggressive effect is unselective, being accompanied by an increase of other behaviors such as avoidance/flee, suggesting an anxiogenic-like activity of the drug. It is concluded that α5/GABA-A receptors might be involved in the modulation of anxiety.

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REFERENCES

Effects of L-655,708 on Social Encounters in Mice


