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Separation-induced ultrasonic vocalization in rat pups: Further pharmacological characterization

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Abstract

In rat pups, ultrasonic vocalizations were emitted in response to separation from the mothers, littermates, and nest. It has been suggested that these separation-induced ultrasonic vocalizations (SIV) in rat pups form one of the animal models of anxiety. The primary aim of the present study is to investigate the effect of the compounds acting on stress-related peptide receptors such as a vasopressin V1b receptor antagonist and a corticotropin-releasing factor CRF1 receptor antagonist in rat pup SIV. The secondary objective is to establish further confirmation of the predictive validity of SIV testing. Both the V1b receptor antagonist SSR149415 and the CRF1 receptor antagonist CP154,526 reduced SIV, suggesting antagonists for stress-related peptide receptors are effective in this model. Furthermore, as with selective serotonin reuptake inhibitors such as fluvoxamine and paroxetine, SIV was also reduced by the serotonin and noradrenaline reuptake inhibitor milnacipran and the metabotropic glutamate receptor 5 antagonist MPEP, while desipramine was without effect. Thus, the present experiment highlights the important role of the stress-related peptide systems as well as of the serotonergic systems in SIV. Moreover, the present data support the usefulness of SIV for evaluating the anxiolytic-like activity of mechanically diverse compounds.

Keywords: Ultrasonic vocalization; Maternal separation; Vasopressin; Corticotropin-releasing factor; Anxiety

1. Introduction

Rat pups, prior to the age of about 14 days, emit a highly stereotyped and well-characterized distress call in the ultrasonic range when separated from their mothers, littermates, and nest. The maternal separation-induced ultrasonic vocalizations (SIV) test has been used as an animal model of anxiety, since the SIV can be dose-dependently suppressed by benzodiazepine (Gardner, 1985; Insel et al., 1986) and non-benzodiazepine anxiolytics (Olivier et al., 1998a; Kehne et al., 1991). The SIV has been viewed as a model offering good predictive validity when assessing the anxiolytic-like potential of compounds, because antidepressants such as selective serotonin reuptake inhibitors (SSRIs), which have been demonstrated to be effective in anxiety disorders, show anxiolytic effects in this model (Borsini et al., 2002). However, neurochemical mechanisms underlying SIV have remained largely unexplored.

It has recently been hypothesized that stress may play a pivotal role in both anxiety and depression. Arginine vasopressin (AVP) and corticotropin-releasing factor (CRF) are key mediators in the neuro-adaptive response to stress (Aguilera and Rabadan-Diehl, 2000; Rivier and Plotsky, 1986). Among their receptor subtypes, V1b and CRF1 receptors have been proposed to mediate these stress responses induced by AVP and CRF, respectively. It has been reported that the CRF subtype 1 receptor (CRF1) antagonist CP154,526 reduced SIV at doses that did not affect general behavior (Kehne et al., 2000). In addition to the CRF system, it has been reported that the AVP receptor 1b (V1b) antagonist SSR149415 produces anxiolytic effects in several animal models of anxiety, particularly models that include highly stressful components (Griebel et al., 2002). Therefore, to investigate the involvement of the stress component in SIV, we investigated the effects of SSR149415 and CP-154,526 in the test.

Our second aim in the present study is to establish further validation of SIV as an animal model of anxiety. It has been

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known that the serotonin and noradrenaline reuptake inhibitor (SNRI) milnacipran is a clinically effective antidepressant (Kasper et al., 1996). In preclinical studies, milnacipran has been found to reduce conditioned fear stress-induced freezing behavior after acute administration (Hascoet et al., 2000), although reports on the anxiolytic effect of milnacipran in animal models of anxiety are limited. The metabotropic glutamate receptor subtype 5 (mGluR5) has been identified as a potential target for anxiety and depression (Spooren et al., 2001). The mGluR5 antagonist MPEP has been reported to exhibit anxiolytic effects in some animal models (Ballard et al., 2005; Brodkin et al., 2002). However, the efficacy of milnacipran and MPEP in SIV in rat pups has not yet been demonstrated. Therefore, in the present study, we also examined the effects of milnacipran and MPEP in SIV to explore the efficacy of mechanically diverse compounds in this model.

2. Materials and methods

2.1. Animals

9- to 11-day-old male and female Sprague-Dawley rat pups (Charles River Laboratories, Yokohama, Japan) weighing 21 to 30 g were used in the following studies. The animals were maintained under a controlled temperature (25±1 °C) and humidity (30–40%), with an inverted 12-h light/dark cycle (lights on at 07:00). There were an approximately equal number of male and female rats in each group. Food and water were available ad libitum except during the test. 8–10 animals were used in each group. All studies were conducted in cooperation with the Taisho Pharmaceutical Co., Ltd. Animal Care Committee and met the Japanese Experimental Animal Research association standards as defined in the Guidelines for Animal Experiments (1987).

2.2. Ultrasonic emissions

Ultrasonic emissions were recorded with an ACO type 7016 microphone, connected to an ACO type 4116 preamplifier (ACO, Co., Ltd., Tokyo, Japan) and a type UMA2 amplifier (Muromachi Co., Ltd., Tokyo, Japan).

2.3. Drugs

SSR149415 (synthesized at Taisho Medicinal Research Laboratories) was dissolved in 5% Cremophor EL. CP154,526 (synthesized at Taisho Medicinal Research Laboratories), diazepam (purchased from Dainippon Pharmaceutical. Co., Osaka, Japan) and MPEP (purchased from Sigma-Aldrich. Co., Tokyo, Japan) were dissolved in 0.3% Tween 80/saline solution. Fluvoxamine, milnacipran (synthesized at Taisho Medicinal Research Laboratories), desipramine (purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan), buspirone (purchased from Sigma-Aldrich. Co., Tokyo, Japan), and paroxetine (purchased from Dongyang Pharmaceutical Chemical Co., Ltd., China) were dissolved in saline. All drugs were injected intraperitoneally (i.p.) at a volume of 10 ml/kg body weight 30 min prior to the test.

2.4. Measurement of SIV

The procedure was adapted from that described by Olivier et al. (1998a). On the day of the experiment, rat pups 9–11 days old were weighed, marked, and injected i.p. with a vehicle or a test compound, 30 min prior to the test. Immediately following the injection, each pup was returned to its mother and littermates until the test. Thirty minutes later the pups were individually placed in 500-ml glass beakers lined with filter paper (ϕ70 mm) located below microphones in sonically isolated boxes ($60 \times 70 \times 60$ cm^3). The number and duration of vocalizations during the 5-min test period was detected using a UMA2 amplifier (Muromachi Co., Ltd., Tokyo, Japan) (settings: high pass=20 kHz, low pass=60 kHz) and a sound pressure of 60 dB was set as the threshold. The beakers were washed between each test, and pups were tested only once. Rectal temperature was recorded immediately following the test session. At the end of the test, the pups were returned to their mothers and littermates.

2.5. Statistics

Data were presented as the mean±S.E.M. Analyses of variance (ANOVA) were used for statistical analysis of ultrasonic vocalization and rectal temperature data. The

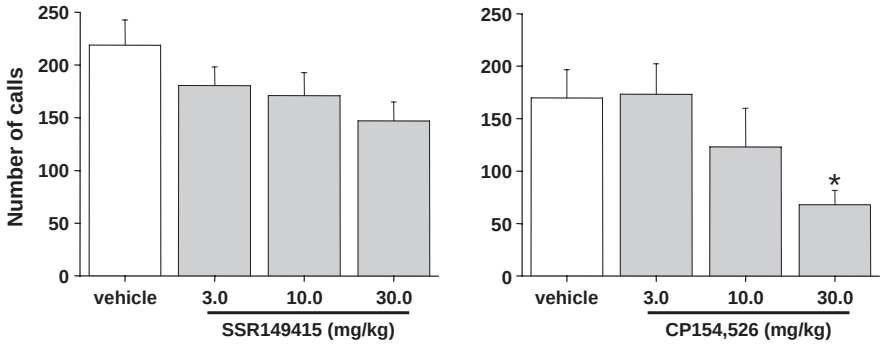

Fig. 1. Effects of the V1b antagonist SSR149415 (3.0, 10.0 and 30.0 mg/kg, i.p.) and the CRF1 antagonist CP154,526 (3.0, 10.0 and 30.0 mg/kg, i.p.) on SIV in rat pups. N=8–10 in each group for SSR149415 and N=8 in each group for CP154,526. Vertical axis displays the number of USV calls in rat pups. All data are expressed as the mean±S.E.M. *p<0.05 indicates significant effects compared to vehicle controls.

Table 1
Effect of the test compounds in the rectal temperature (°C) on SIV in rat pups

Compound	Vehicle	Low dose	⟶	High dose
SSR149415	34.16 (±0.134)	34.58 (±0.236)	34.37 (±0.127)	34.32 (±0.222)
CP154,526	34.23 (±0.487)	34.18 (±0.298)	33.31 (±0.496)	33.13 (±0.396)
Fluvoxamine	34.64 (±0.233)	34.73 (±0.169)	34.65 (±0.160)	34.68 (±0.251)
Paroxetine	35.53 (±0.273)	35.19 (±0.247)	35.39 (±0.223)	35.16 (±0.157)
Milnacipran	34.97 (±0.347)	34.97 (±0.135)	34.54 (±0.153)	34.17* (±0.218)
Diazepam	34.44 (±0.080)	34.12 (±0.162)	33.78 (±0.294)	33.76 (±0.200)
Buspirone	35.27 (±0.197)	34.00* (±0.471)	33.50** (±0.274)	32.90** (±0.298)
MPEP	34.79 (±0.202)	34.84 (±0.237)	34.33 (±0.213)	34.66 (±0.204)
Desipramine	35.61 (±0.123)	35.86 (±0.076)	35.33 (±0.146)	35.17 (±0.206)

All data are expressed as the mean (±S.E.M.). *$p<0.05$ and **$p<0.01$ indicate significant effects compared to vehicle controls.

parametric Dunnett's test was performed on the different sets of data.

3. Results

3.1. Effect of CRF1 and V1b antagonists on SIV in rat pups

It was observed that SSR149415 [$F(3,34)=2.04$, $p=0.126$] had a tendency to reduce SIV, although it had not reached statistically significance (Fig. 1). In contrast, the SSR149415 treatment group showed no effects in rectal temperature (Table 1). CP154,526 [$F(3,28)=3.11$, $p<0.05$] dose-dependently and significantly reduced SIV compared with vehicle groups (Fig. 1) without affecting rectal temperature (Table 1).

3.2. Effect of several antidepressants and anxiolytics on SIV in rat pups

The effects of SSRIs (fluvoxamine and paroxetine) and SNRI (milnacipran) on SIV are shown in Fig. 2. Fluvoxamine [$F(3,28)=15.97$, $p<0.01$] and paroxetine [$F(3,36)=10.19$, $p<0.01$] significantly reduced SIV compared with vehicle groups. Significant reductions were noted with lower doses of both drugs (1.0 and 0.3 mg/kg, respectively). Likewise, milnacipran [$F(3,36)=8.00$, $p<0.01$] dose-dependently and significantly reduced SIV, although hypothermic effect was observed at the highest dose (Table 1). The effect of milnacipran on USVs was less potent than the effects of fluvoxamine and paroxetine.

Anxiolytic benzodiazepine diazepam [$F(3,28)=11.41$, $p<0.01$] and the serotonin-1A receptor partial agonist, buspirone [$F(3,32)=19.47$, $p<0.01$], dose-dependently reduced SIV (Fig. 3). However, buspirone reduced rectal temperature (Table 1).

The effects on the SIV of the mGluR5 antagonist MPEP and those of the tricyclic antidepressant/noradrenaline uptake inhibitor desipramine are shown in Fig. 4. MPEP [$F(3,36)=7.96$, $p<0.01$] significantly and potently reduced SIV. In contrast, desipramine [$F(3,36)=0.95$, $p=0.43$] failed to decrease SIV production. Neither MPEP nor desipramine affected rectal temperature at doses tested in this study (Table 1).

4. Discussion

In the present study, we demonstrated that the V1b antagonist SSR149415 and the CRF1 antagonist CP154,526 reduced SIV in rat pups without hypothermic effects. It has been suggested that stress responses are triggered by alterations in the secretion of neuropeptides (Van de Kar and Blair, 1999), and that both AVP and CRF are key factors in mediating neurochemical changes in response to exposure to various stresses (Rivier and Plotsky, 1986; Aguilera and Rabadan-Diehl, 2000). It has been reported that a V1b antagonist and a CRF1 antagonist attenuate the stress-induced increase in the release of ACTH (Heinrichs et al., 2002; Serradeil-Le Gal et al., 2002); this attenuation is associated with the anxiolytic effects of these substances. It has also been reported that both V1b antagonists and CRF1 antagonists exhibit anxiolytic effects particularly in models involving highly stressful situations (Griebel et al., 2002; Chaki et al., 2004). Based on these findings and the present results, it has been suggested that the SIV test is an appropriate anxiety model for stressful situations and that this model is useful for evaluating the anti-

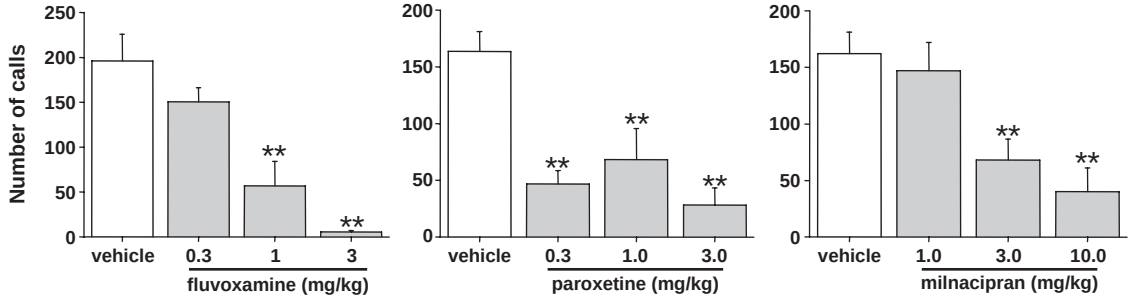

Fig. 2. Effects of the SSRIs fluvoxamine (0.3, 1.0, and 3.0 mg/kg i.p.), paroxetine (0.3, 1.0, and 3.0 mg/kg, i.p.), and the SNRI milnacipran (1.0, 3.0 and 10.0 mg/kg, i.p.) on SIV in rat pups. $N=8$ in each group for fluvoxamine, $N=10$ in each group for paroxetine and milnacipran. Vertical axis displays the number of USV calls in rat pups. All data are expressed as the mean±S.E.M. **$p<0.01$ indicates significant effects compared to vehicle controls.

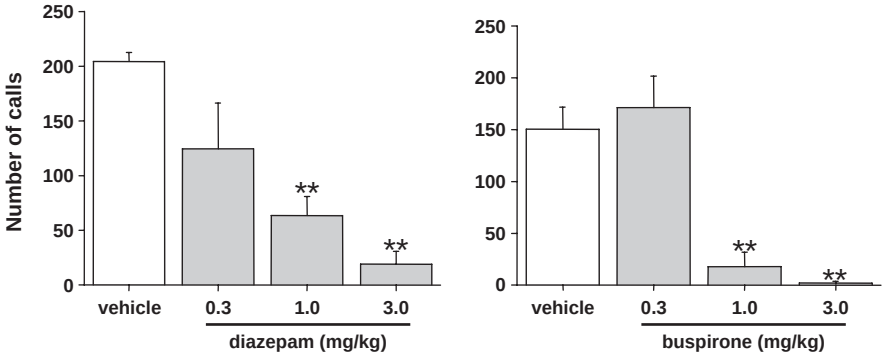

Fig. 3. Effects of the anxiolytic benzodiazepine diazepam (0.3, 1.0, and 3.0 mg/kg, i.p.) and serotonin-1A partial agonist buspirone (0.3, 1.0, and 3.0 mg/kg, i.p.) on SIV in rat pups. $N=8$ in each group for diazepam and $N=9$ in each group for buspirone. Vertical axis displays the number of USV calls in rat pups. All data are expressed as the mean ± S.E.M. $^{**}p<0.01$ indicates significant effects compared to vehicle controls.

stress/anxiolytic effects of compounds acting on stress-related peptide receptors such as V1b antagonists and CRF1 antagonists. Contrary to the present study, it has been reported that stress (exposure to unfamiliar adult male rats) reduced SIV (Takahashi, 1992), which may represent a protective behavioral strategy. However, a clear reduction in SIV by stress exposure did not occur until 12 days of age. Given that 9- to 11-day-old rat pups were used in this study, this may not be the case in the present results. It should be noted that it has been reported that intracerebroventricularly injected CRF reduced SIV in mouse pups (Dirks et al., 2002). The same study reported that CRF level and SIV are related in a curvilinear fashion and that the effect of CRF depends on the levels of stress. Thus, the stress level in the current condition may be sufficient to detect the anti-stress effect of the compounds.

The secondary aim of the present study is to establish further confirmation of the predictive validity of the SIV test using mechanically different antidepressants and anxiolytics. In the present study, clinically prescribed anxiolytics such as a benzodiazepine (diazepam) and a serotonin-1A receptor partial agonist (buspirone) potently decreased SIV in rat pups, a finding consistent with previous reports (Olivier et al., 1998a,b). Moreover, SSRIs such as paroxetine and fluvoxamine markedly reduced SIV at lower doses compared to other animal models of anxiety, as observed in a previous study (Olivier et al., 1998a; Winslow and Insel, 1990). In contrast, as reported previously (Winslow and Insel, 1990; Kehne et al., 2000), we found that the tricyclic antidepressant/noradrenaline uptake inhibitor desipramine in fact failed to decrease SIV in rat pups. This observation is of interest in light of clinical findings demonstrating that SSRIs are clinically effective in treating anxiety disorders, now becoming the first-line treatment for such disorders (Schatzberg, 2000), while noradrenaline uptake inhibitors may be less effective or ineffective (Dow and Kline, 1997; Figgitt and McClellan, 2000). Thus, the present study confirmed the predictive validity of the SIV test as an animal model of anxiety to assess the anxiolytic-like potential of compounds. It should be noted that most classical anxiety models (such as the elevated plus-maze task) are incapable of detecting the anxiolytic-like activity of serotonergic agents such as SSRIs and serotonin-1A receptor agonists (Borsini et al., 2002).

Interestingly, milnacipran, an SNRI (Moret et al., 1985), produced anxiolytic effects in SIV testing. It has been reported that milnacipran exhibits anxiolytic effects in the conditioned fear stress test (Hashimoto et al., 1996; Miyamoto et al., 2004) and the four-plate test (Hascoet et al., 2000). It should be noted that this is the first study to examine the anxiolytic-like potential of an SNRI in an SIV test, and that the anxiolytic effect on SNRI was further confirmed in this paradigm. Moreover, the effectiveness of SNRIs in treating anxiety disorders has recently been established (Katzman, 2004; Silverstone, 2004), and venlafaxine, an SNRI, has been approved for generalized anxiety disorder. Therefore, the

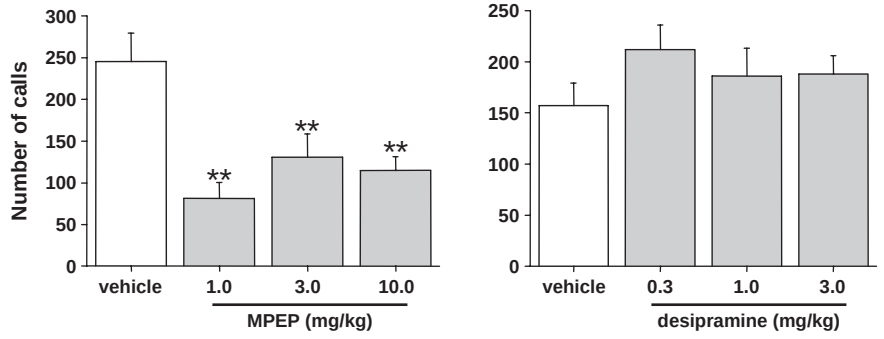

Fig. 4. Effects of the mGluR5 antagonist MPEP (1.0, 3.0, and 10.0 mg/kg, i.p.) and antidepressant desipramine (0.3, 1.0, and 3.0 mg/kg, i.p.) on SIV in rat pups. $N=10$ in each group for MPEP and desipramine. Vertical axis displays the number of USV calls in rat pups. All data are expressed as the mean ± S.E.M. $^{**}p<0.01$ indicates significant effects compared to vehicle controls.

present results further support the predictive validity of the SIV test as an anxiety model, and, conversely, it is suggested that milnacipran may offer clinical efficacy in the treatment of anxiety disorders. In the above-mentioned anxiety models, it has been reported that SSRIs exhibit more potent anxiolytic effects than SNRIs (Hashimoto et al., 1996; Hascoet et al., 2000; Miyamoto et al., 2004). Consistent with these findings, we found that milnacipran was less effective in reducing SIV than SSRIs. Thus, it is conceivable that serotonergic transmission might play an important role in exhibiting anxiolytic effects, and it may be said that the balance between serotonin and noradrenaline seems to be a crucial factor in producing anxiolytic effects in these anxiety models; this may explain the reason for desipramine's lack of anxiolytic effect.

We further investigated the utility of this model by testing a putative anxiolytic, an mGluR5 antagonist. It has been reported that dysfunction of glutamatergic transmission is involved in anxiety and that NMDA receptor antagonists exhibit anxiolytic effects in a variety of animal models of anxiety (Spooren et al., 2001; Brodkin et al., 2002), including the SIV test (Kehne et al., 1991). In the present study, the mGluR5 antagonist MPEP significantly reduced SIV in rat pups. These findings suggest that glutamatergic transmission plays a role in the SIV model and that this model is suitable to evaluate the anxiolytic-like activity of compounds acting on the glutamate system. Recently, it has been demonstrated that MPEP reduces stress-induced increases in extracellular noradrenaline release in the frontal cortex (Page et al., 2005). Therefore, the reduction of stress-induced noradrenaline release may be, at least in part, involved in the anxiolytic effects of MPEP in this model.

In summary, the present results suggest that a stress component might be involved in the emission of ultrasonic vocalizations in rat pups, induced by separation from their dams and littermates, and that the SIV test is useful in the assessment of the anti-stress/anxiolytic effects of novel agents acting through regulation of neuropeptides. Moreover, the present study revealed that the SIV test could also be used to evaluate the anxiolytic-like potential of SNRIs as well as mGluR5 antagonists. The advantages of the SIV test in rat pups are its high throughput and easy screening of test batteries for a candidate anxiolytic. Further studies are required to elucidate the mechanisms underlying SIV and the sites of action of the anxiolytics in this model. The disadvantage of this model is that experiments involving chronic administration cannot be performed by using pre-weaning rat pups. The reason for the finding is limited time during which a vocalization response from separation. Nevertheless, the SIV test in rat pups can be successfully used and adopted as an accepted procedure for detecting the anti-stress/anxiolytic-like potential of compounds featuring a wide range of mechanisms.

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