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Duration of ultrasonic vocalizations in the isolated rat pup as a behavioral measure: Sensitivity to anxiolytic and antidepressant drugs

Robert A. Hodgson *, Donald H. Guthrie, Geoffrey B. Varty

Department of Neurobiology, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

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Abstract

Neo-natal rats emit ultrasonic vocalizations (USVs) when isolated from their mothers and littermates. Clinically effective anxiolytics reliably reduce USVs, making this behavior a useful animal model of the anxiolytic potential of novel pharmacological approaches to the treatment of anxiety. Here, we assess the hypothesis that USV duration (total time spent vocalizing) is a more sensitive measure of anxiolytic and antidepressant efficacy than USV number by testing established and putative anxiolytics in this model. Negative geotaxis and righting reflex latency were measured to assess sedating properties. The benzodiazepines, CDP (1–10 mg/kg) and diazepam (0.3–3 mg/kg), the $5HT_{1A}$ partial agonist, buspirone (0.3–3 mg/kg), and the mGluR5 antagonist, MTEP (1–30 mg/kg), reduced USV duration at lower doses and to a greater magnitude than USV number. The benzodiazepines, unlike buspirone and MTEP, produced measurable sedation, but it was dissociable from reductions in USV duration. The SSRI antidepressants, fluoxetine (1–30 mg/kg) and citalopram (0.3–30 mg/kg), reduced USV duration more than number with no measurable effect on sedation. The tricyclic antidepressants, imipramine (1–10 mg/kg) and amitriptyline (1–30 mg/kg), had no effect dissociable from sedation. These data support USV duration as a more sensitive and useful measure than USV number in the isolated rat pup model.

Keywords: Anxiety; USV; Benzodiazepine; Depression; mGluR5; 5HT1A; SSRI; Tricylic

1. Introduction

Rat pups reliably emit ultrasonic vocalizations (USVs) when separated from their mothers and littermates (Insel et al., 1986). This appears to be designed to elicit a retrieval response from the mothers (Allin and Banks, 1972) on whom the pups rely for survival. The number (or rate) of pup vocalizations during a period of maternal separation, is thought to reflect the degree of anxiety the animals are experiencing — the greater the number of USVs, the greater the level of anxiety (Winslow and Insel, 1991). This is supported by evidence that manipulations designed to increase or decrease anxiety can affect USV rate. For example, exposure to either unfamiliar odors (Oswalt and Meier, 1975), or to cold (Allin and Banks, 1972), produce an increase in USV rate; whereas returning a pup to a familiar maternal odor reduces USV rate (Hofer and Shair, 1980). Additionally, pups from a high-anxiety strain produce significantly more USVs than those from a low-anxiety strain (Naito et al., 2000), a finding that further substantiates the link between USVs and anxiety.

Rat pup USVs are easily inducible, by separating a pup from its mother, and are easily quantifiable. Therefore, rat pup USVs have been used as a useful animal model of anxiety-like behavior. This model is especially useful for the development of novel pharmacotherapies for the treatment of anxiety in man. Numerous pharmacological agents known to be anxiolytic in man have been tested in this model and have been shown to reduce USV number. Examples of such compounds are benzodiazepines (Olivier et al., 1998b), and partial $5HT_{1A}$ receptor agonists (Kehne et al., 1991; Olivier et al., 1998a). Additionally, compounds that are active in other animal models predictive of anxiolytic efficacy are also active in the rat pup model. Examples of these compounds include the metabotropic

* Corresponding author. K-15-2600, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA. Tel.: +1 908 740 3276; fax: +1 908 740 3294.
E-mail address: Robert.Hodgson@SPCorp.com (R.A. Hodgson).

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glutamate 5 receptor (mGluR5) antagonists, MTEP (Iijima and Chaki, 2005) and MPEP (Brodkin et al., 2002), the corticotropin releasing factor CRF1 receptor antagonist, CP-154,526 (Kehne et al., 2000; Hodgson et al., 2007) and the vasopressin V1b receptor antagonist, SSR149415 (Hodgson et al., 2007). As such, the rat pup USV model has been used to measure of the pre-clinical anxiolytic potential of novel chemical entities.

Aside from the number of USVs a separated pup emits during a period of maternal separation, there are other aspects of the calling behavior that are variable, which therefore, could be used to communicate the degree of distress. One such factor is the USV duration. For example, a single sustained USV of 500 ms likely expresses an affective state that is different from a single sustained USV of 250 ms. In this hypothetical example, USV number is equivalent; only by measuring USV duration reflects the difference in affective state apparent. Extending this logic beyond a single USV to a period of maternal separation during which a pup is emitting numerous USVs, lowering a pup's state of anxiety would result in a reduction in the total time a separated pup spends calling, assuming no decrease in the number of USVs. Additionally, if a pup were to lower the number of USVs during a period of separation, without decreasing the average duration, there would be a lowering of the total time spent calling. Therefore, measuring the total duration that a separated pup spends calling should be a more sensitive measure of reductions in anxiety than simply counting the calls.

To assess our hypothesis we tested both established and putative anxiolytics in the model and measured both USV number and duration for head-to-head comparisons. Drugs tested were the benzodiazepines, chlordiazepoxide (CDP) and diazepam, the $5HT_{1A}$ partial agonist buspirone, and the mGluR5 antagonist MTEP. Previously, we have tested the nociceptin NOP1 receptor agonist Ro64-6198 (Varty et al., 2005), the CRF1 receptor antagonist CP-154,526, the V1b receptor antagonist SSR149415 (Hodgson et al., 2007) in this model and have found that USV duration is more sensitive to the anxiolytic effect of these compounds. In other words, total USV duration was significantly reduced at lower doses than USV number. Further, we employed sedation tests typically used in this assay (negative geotaxis and righting reflex latency), to determine whether drug effects on USV duration were independent of the sedative effects.

In addition, because less is known about the sensitivity of this assay to antidepressants, we assessed the utility of the USV duration measure with regard to assessing antidepressant efficacy. To do this, we tested two SSRIs (citalopram and fluoxetine) and two tricyclics (amitriptyline and imipramine) and compared the USV number and duration measures.

To assess the sensitivity of the model to non-pharmacological manipulations in the anxiety level of the pups, we tested animals either at room temperature or on a cold plate lowering the ambient temperature to 10 °C. Decreasing the temperature of the pups has previously been shown to increase USVs (Olivier et al., 1998a,b). Cooling the pups should also impair performance on the sedation measures. Unlike the pharmacological manipulations, exposure to cold is predicted to produce opposing effects on sedation (impairment) and USV rate (increase), making them easily dissociable.

Since the model's inception, USV number has been the standard measure of both pharmacological and non-pharmacological manipulations of anxiety and, to our knowledge, no one has attempted to determine if another rat pup USV metric is sensitive to the potential of novel anxiolytics. Collectively these studies provide a data set that allows for a comparison of the sensitivity of USV duration and USV number as measures of an isolated rat pup's stress level. Moreover, by measuring the sedation levels of the animals within the same studies, we are able to determine the degree to which the effects of both pharmacological and non-pharmacological manipulations affect USVs in a manner independent of sedation.

2. Materials and methods

2.1. Animals

Pregnant female CD rats (gestation day 9) were delivered from Charles River Labs (Kingston, NY). The animals were housed in a climate controlled vivarium on a 12 hour light/dark cycle (07:00–19:00) with food and water available ad libitum. Twenty-four to 48 h after birth, each litter was culled or fostered to $n=10$ to control for potential differences in maternal care. Male and female rat pups (8–10 days post natal: body weight range 17–30 g) were used in all studies. Animal care and testing procedures were conducted in conformity with the Schering-Plough Animal Care and Use Committee, and in compliance with the NIH "Guide to the Care and Use of Laboratory Animals" and the Animal Welfare Act.

2.2. Measurement of ultrasonic vocalizations (USV)

Ultrasonic microphones (Mini-3 Bat Detector; Ultra Sound Advice, London, UK) converted the vocalizations to audible sounds. Using these audible vocalizations, the microphones were tuned to optimize recording for each animal by the experimenter to an average frequency of approximately 42 kHz. This frequency produced the clearest audible signal. The DC output from the bat detector was linked by standard microphone cable to an adjustable audio filter which converted the audio signal to a digital signal. The audio filter was linked to a computer equipped with UltraVox 2.0 software (Noldus Information Technology, Wagenigen, Netherlands). The software recorded the time of the onset and offset of each vocalization which allowed for the determination of USV number and duration. Number represented a count of USVs made during the testing session; duration represented the cumulative time a pup spent vocalizing during the testing session.

2.3. Testing procedure

There is a high degree of inter-animal variability in calling behavior. To minimize the impact this variability had on our studies, the pups were pre-screened 24 h prior to testing and assigned to balanced groups of high and low responders. On the

test day, animals were injected with the appropriate compound/dose and returned to their home cage with their littermates and dam. Following the appropriate pre-treatment time, each pup was placed in a 500 ml glass beaker, which was placed in a sound attenuating chamber (H: 60 cm; W: 63 cm; D: 40 cm). USVs were recorded for 10 min by ultrasonic microphones mounted on top of each beaker. Each drug was tested separately in a naïve cohort and a between-groups design was used for all compounds.

In the cold condition, an aluminum tray was placed inside the sound attenuating chambers and ice was placed on the tray. The beakers were then placed on the tray such that they did not have any direct contact with the ice. A probe situated at the bottom of the beaker was used to measure temperature and pups were placed inside the beaker when the temperature had dropped to 10 °C. This process was repeated for each animal tested with a new beaker and tray. Temperatures inside the warm beakers measured approximately 20 °C, which was also the temperature at which all drug studies were conducted.

2.4. Measurement of motor function

Motor function assessment was carried out immediately following USV recording using two tests. To assess negative geotaxis latency, a pup was placed with its head facing downward on an inclined plane (25° from base). The latency for the pup to turn 90° and reach a position parallel with the bottom and top edges of the plane was recorded. Latency recordings were truncated at 30 s. To assess righting reflex latency, a pup was placed flat on its back and the latency to reach an upright position, with all four paws flat on the surface, was recorded. Animals were given a maximum of 10 sec to complete the task.

2.5. Drugs

All drugs were obtained from Sigma (St. Louis, MO) except MTEP (synthesized by the SPRI Medicinal Chemistry Department). MTEP was suspended in 10% Tween 80; all other drugs were dissolved in saline. All drugs were delivered intraperitoneally (ip) 30 min prior to testing and all doses are expressed as free base.

2.6. Method of statistical analysis

For all drug studies, USV number, USV duration, negative geotaxis and righting reflex data were analyzed using one-way analysis of variance (ANOVA). A Dunnett's test comparing all dose groups against the control group was employed as a post-hoc method of analysis to determine statistically significant differences of individual doses. For the cold plate study, data were analyzed using a *t*-test. Significance was defined as $p<0.05$.

3. Results

3.1. Anxiolytics

CDP, tested at 1, 3, and 10 mg/kg, dose-dependently reduced USV number [$F(3,56)=9.23$, $p<0.01$] and duration [$F(3,56)=11.96$, $p<0.01$]. Post-hoc tests revealed significant reductions in USV number (3 and 10 mg/kg), and USV duration (1, 3 and 10 mg/kg), relative to vehicle. At the minimum effective dose of 1 mg/kg, USV number was inhibited by 18% and USV duration was inhibited by 28% relative to vehicle (Fig. 1A). CDP increased the latency to complete the negative geotaxis [$F(3,56)=6.22$, $p<0.01$] and righting reflex tasks [$F(3,56)=7.29$, $p<0.01$]. In both cases, only the 10 mg/kg group had significantly longer latencies to complete the tasks relative to the vehicle group (Fig. 1B).

The other benzodiazepine, diazepam, tested at 0.3, 1 and 3 mg/kg, dose-dependently reduced USV number [$F(3,36)=4.19$, $p<0.05$] and USV duration [$F(3,36)=10.21$, $p<0.01$]. Post-hoc tests revealed significant reductions in USV number (3 mg/kg), and USV duration (1 and 3 mg/kg) relative to vehicle control. At the minimum effective dose of 1 mg/kg, USV number was inhibited by 19% whereas USV duration was inhibited by 50% (Fig. 1C). Diazepam also impaired both negative geotaxis [$F(3,36)=7.45$, $p<0.01$] and righting reflex [$F(3,36)=8.21$, $p<0.01$]. In both cases, the 1 and 3 mg/kg dose groups were significantly impaired relative to vehicle (Fig. 1D).

Buspirone, tested at 0.3, 1, and 3 mg/kg, dose-dependently reduced both USV number [$F(3,36)=4.46$, $p<0.01$] and duration [$F(3,36)=15.41$, $p<0.01$]. Using both measures, all three doses reduced calling relative to vehicle. The minimum effective dose (0.3 mg/kg) inhibited USV number by 44% and duration by 68% (Fig. 1E). Buspirone had no effect on either negative geotaxis [$F(3,36)=0.09$, $p>0.05$] or righting reflex latency [$F(3,36)=1.18$, $p>0.05$] relative to vehicle (Fig. 1F).

MTEP, tested at 1, 3, 10, and 30 mg/kg, significantly lowered both USV number [$F(4,45)=2.87$, $p<0.05$] and USV duration [$F(4,45)=7.08$, $p<0.01$]. Post-hocs demonstrated that all four doses significantly lowered USVs on both measures relative to vehicle. At 1 mg/kg USV number was inhibited by 27% and USV duration was inhibited by 30% (Fig. 1G). MTEP had no significant effect on either negative geotaxis [$F(4,45)=0.42$, $p>0.05$] or righting reflex latency [$F(4,45)=1.18$, $p>0.05$] relative to vehicle (Fig. 1H).

3.2. Antidepressants

The SSRI antidepressant, citalopram, tested at 0.3, 1, 3, 10, and 30 mg/kg, significantly reduced USV number [$F(5,54)=2.71$, $p<0.05$] and duration [$F(5,54)=5.80$, $p<0.01$]. Post-hoc analysis demonstrated that the 30 mg/kg dose significantly lowered USV number, whereas all five tested doses lowered USV duration. The lowest effective dose (0.3 mg/kg) inhibited USV number by 7% and USV duration by 47% relative to vehicle (Fig. 2A). There was no effect on either negative geotaxis [$F(5,54)=0.36$, $p>0.01$] or righting reflex latency [$F(5,54)=1.26$, $p>0.05$] relative to vehicle (Fig. 2B).

The other SSRI antidepressant, fluoxetine, tested at 1, 3, 10, and 30 mg/kg, reduced USV number [$F(4,45)=3.27$, $p<0.05$] and duration [$F(4,45)=5.44$, $p<0.01$]. A post-hoc test demonstrated that there was a significant effect on USV number in the 30 mg/kg group, and on duration in the 3 and 30 mg/kg groups. At the minimum effective dose (3 mg/kg) there was a 24% reduction in USV number and a 56% reduction in USV duration

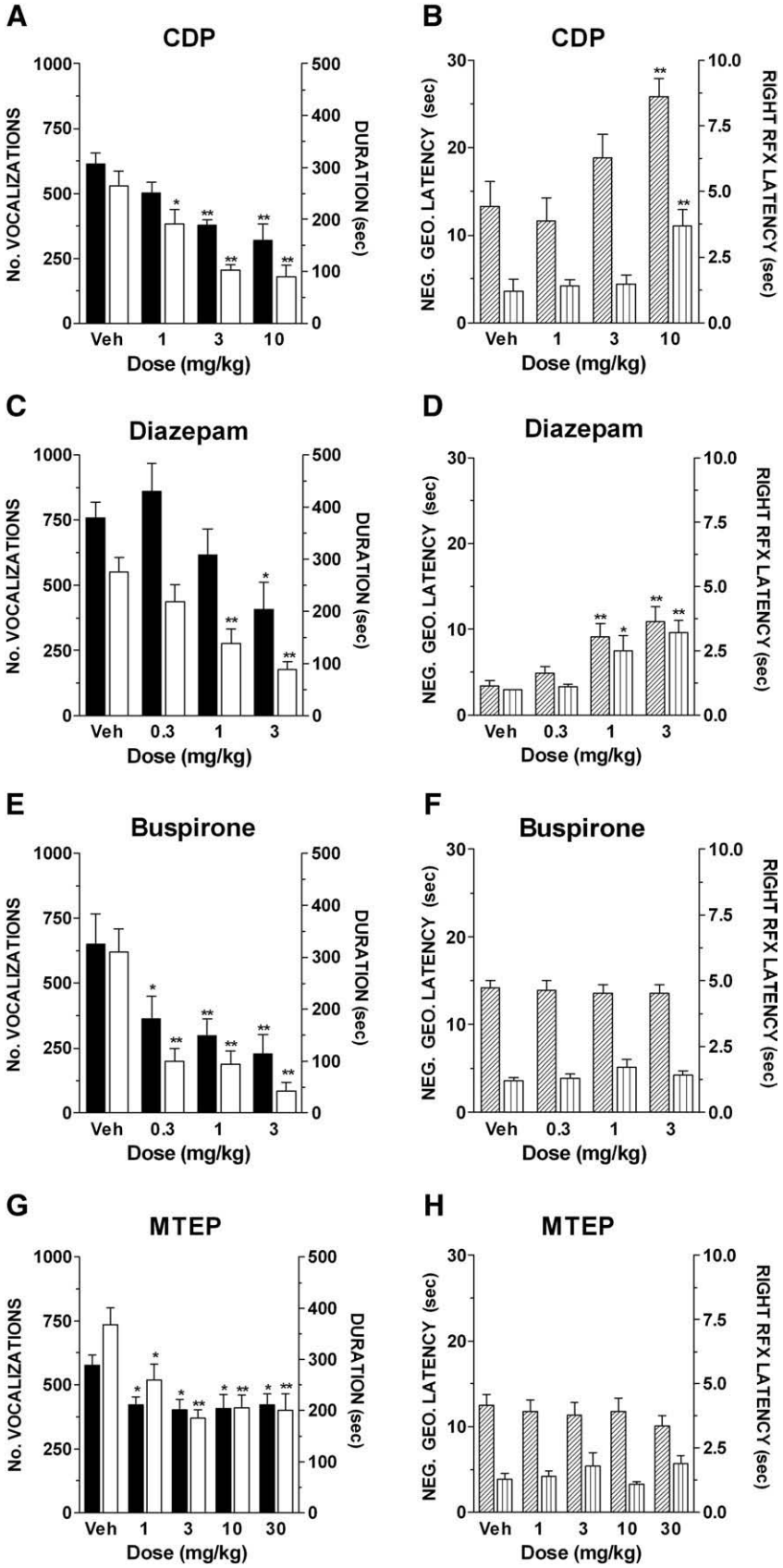

Fig. 1. Effect of anxiolytic agents on rat pup USVs. The effect of four different established and putative antidepressants on USV number (black bars), USV duration (open bars), negative geotaxis (diagonal stripes), and righting reflex (vertical stripes). The four compounds tested, CDP (A), diazepam (C), buspirone (E) and MTEP (G) were efficacious. CDP (B), diazepam (D) and MTEP (H) produced measurable sedation in the model; buspirone (F) did not. The bars represent group ($n=10$) means±SEM. * represents $p<0.05$ relative to vehicle; ** represents $p<0.01$ relative to vehicle.

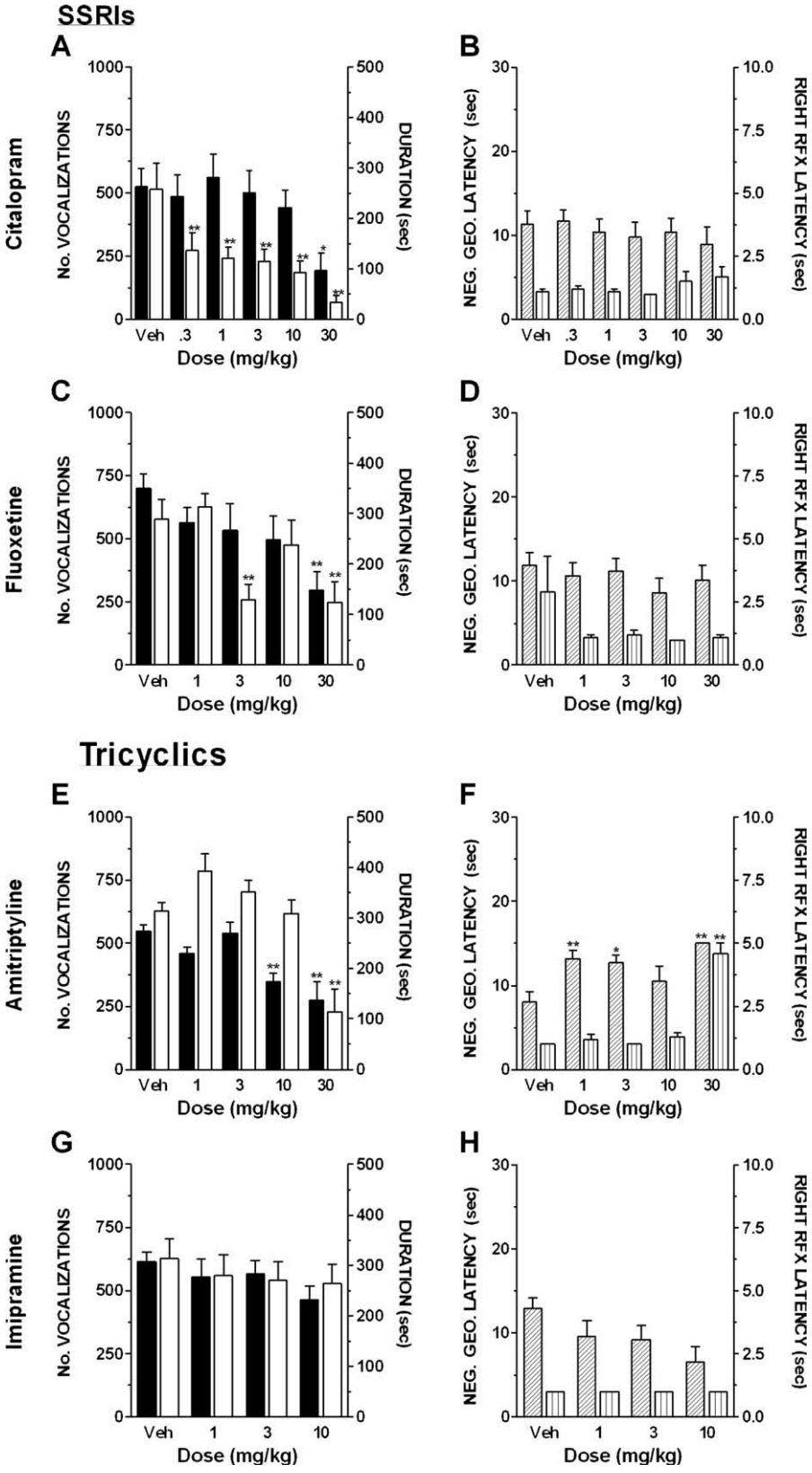

Fig. 2. Effect of Antidepressants on Rat Pup USVs. The effect of four antidepressants on USV number (black bars), USV duration (open bars), negative geotaxis (diagonal stripes), and righting reflex (vertical stripes). The two SSRIs tested, citalopram (A), and fluoxetine (C) were efficacious in the model with no measurable sedation (B and D respectively). Amitriptyline significantly reduced both call number and duration (E), but also impaired negative geotaxis and righting reflex (F). Imipramine had no significant effect on either USV measure (G) or either sedation measure (H). The bars represent group ($n=10$) means ± SEM. * represents $p<0.05$ relative to vehicle; ** represents $p<0.01$ relative to vehicle.

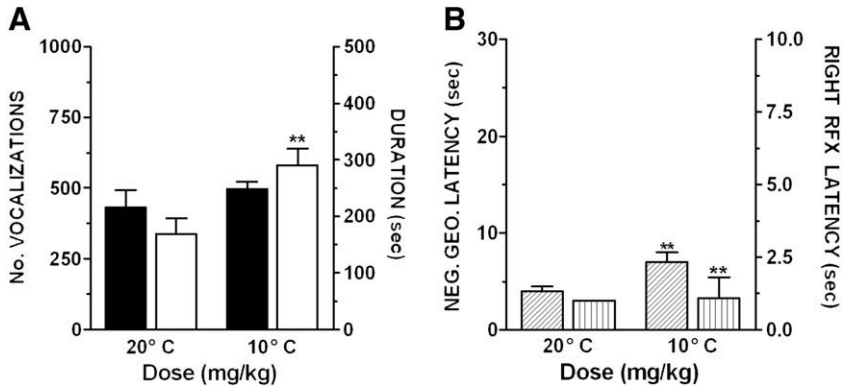

Fig. 3. Effect of cold on rat pup USVs. The effect of reducing ambient temperature (20 °C) on separation-induced USVs. A. The duration (open bars), but not the number (black bars) increase in response to the cold condition (10 °C). B. Cooling the pups impairs both negative geotaxis (diagonal lines) and righting reflex (vertical lines). The bars represent group ($n=10$) means±SEM. * represents $p<0.05$ relative to vehicle; ** represents $p<0.01$ relative to vehicle.

(Fig. 2C). Fluoxetine had no effect on either negative geotaxis [$F(4,45)=0.61$, $p>0.05$] or righting reflex latency [$F(4,45)=1.54$, $p>0.05$] relative to vehicle (Fig. 2D).

The tricyclic antidepressant, amitriptyline, tested at 1, 3, 10, and 30 mg/kg, significantly reduced both USV number [$F(4,45)=7.13$, $p<0.01$] and duration [$F(4,45)=12.26$, $p<0.01$]. Both 10 and 30 mg/kg significantly reduced USV number relative to vehicle; however only the 30 mg/kg group reduced had USV duration. At 10 mg/kg, USV number was inhibited by 36% and USV duration was inhibited by 2%. At 30 mg/kg, USV number was inhibited by 50% and USV duration was inhibited by 64% (Fig. 2E). Amitriptyline significantly increased both negative geotaxis [$F(4,45)=5.31$, $p<0.01$] and righting reflex latency [$F(4,45)=24.32$, $p<0.01$]. The 1, 3, and 30 mg/kg were significantly impaired on the negative geotaxis measure and the 30 mg/kg group was significantly impaired on the righting reflex measure (Fig. 2F).

The other tricyclic, imipramine, tested at 1, 3, and 10 mg/kg had no significant effect on either USV number [$F(3,36)=1.32$, $p>0.05$] or duration [$F(3,36)=0.33$, $p>0.05$] relative to vehicle (Fig. 2G). Imipramine had no effect on either negative geotaxis [$F(3,36)=2.39$, $p>0.05$] and righting reflex latency [$F(3,36)=0.00$, $p>0.05$] relative to vehicle (Fig. 2H). A higher dose of imipramine was not tested because pilot work indicated that 30 mg/kg produced marked sedation in the pups.

3.3. Cold plate

When comparing the warm and cold conditions, exposing the pups to the cold significantly increased USV duration [$t(38)=3.09$, $p<0.01$]; whereas the increase in USV number was not significant [$t(38)=1.01$, $p>0.05$]. USV duration was increased by 73% and USV number was increased by 15% (Fig. 3A). Both negative geotaxis [$t(38)=6.41$, $p<0.01$] and righting reflex [$t(38)=5.97$, $p<0.01$] latencies were impaired by exposure to the cold plate (Fig. 3B).

4. Discussion

We tested four compounds (CDP, diazepam, buspirone, and MTEP), with proven efficacy in rodent models of anxiety-like behavior. All reduced USV number in isolated rat pups, which is consistent with the published literature (Kehne et al., 1991; Olivier et al., 1998b; Brodkin et al., 2002; Iijima and Chaki, 2005). Furthermore, we found that USV duration was consistently more sensitive to the anxiolytic-like effects of the compounds. In each case the lowest dose that significantly lowered USV duration was as low, or lower than, the lowest dose required to significantly reduce USV number. Moreover, for each of these compounds, the lowest effective dose produced a greater percent inhibition of call duration than of call number. The increased sensitivity of the duration we found in these experiments is consistent with previously published data from our lab using different classes of putative anxiolytics in the rat pup USV model. Specifically, we have previously demonstrated that duration is more sensitive to the anxiolytic-like effects of the CRF1 receptor antagonist CP-154,526 the V1b receptor antagonist SSR149415 (Hodgson et al., 2007) and the nociceptin (NOP1) receptor agonist Ro64-6198 (Varty et al., 2005).

Sedative properties have been reported for each of the four anxiety-lowering compounds we tested, CDP (Greeley and Cappell, 1985), diazepam (Kalynchuk and Beck, 1992), buspirone (Vaidya et al., 2005) and MTEP (Varty et al., 2005). Therefore, we carefully assessed the possibility that reduction in USV duration was a product of the sedative effects of these drugs by measuring negative geotaxis and righting reflex latency immediately following recording USVs. In each case, the minimum effective dose (using the duration measure) produced no sedation. Thus, relative to the side effects we assessed, the USV duration measure produced a greater therapeutic window.

SSRIs have been reported to be active in the rat pup USV model (e.g., Winslow and Insel, 1990; Kehne et al., 2000). Tricyclics have been reported to be active (e.g., Olivier et al., 1998b; Podhora and Brown, 2000), inactive (e.g., Gardner, 1985), or even anxiogenic (e.g., Winslow and Insel, 1990; Kehne et al., 2000). Although not tested in these studies, SNRI antidepressants such as venlafaxine have been shown to reduce separation-induced vocalizations in rodents (Rupniak et al. 2000, Fish et al., 2004). For a review of antidepressant activity

in the rat pup USV and other models of anxiety see Borsini et al. (2002). Our results with antidepressants were also mixed. In our hands, the model was sensitive to the effects of both SSRIs tested (fluoxetine and citalopram) and in both cases duration was more sensitive to the drug effect. Data from the tricyclic antidepressants were more complicated. Imipramine had no effect in the model. It is possible that had we gone to higher doses we would have found an effect; however pilot work indicated that a dose of 30 mg/kg produced overt sedation in the pups, which would have made it difficult to test. Amitriptyline significantly reduced both USV number and duration, and was the only drug tested that had a larger effect on USV number than duration (as measured by the minimum effective dose). However, we also found a significant reduction in righting reflex latency at a dose that was lower than the minimum efficacious dose. It is therefore impossible to dissociate the effect on USVs from sedation, making the amitriptyline data impossible to interpret.

The superior efficacy of SSRIs in the model, likely reflects the fact that SSRIs are more effective in treating anxiety than tricyclics as measured by the number of types of anxiety (Zohar and Westenbert, 2000) or by their more rapid onset of action (Nutt, 2000). More work is required to assess the utility of this model with regard to assessing the potential of putative antidepressants.

In the only non-pharmacologic manipulation we conducted, we found that lowering the ambient temperature from 20° to 10° increased the USV rate of the pups. Exposure to cold has previously been shown to increase USV number (Olivier et al., 1998a,b), which likely reflects increased anxiety in that the cold condition makes the absence from the mother's thermo-regulating effects more salient. As was the case with the pharmacological manipulations, we found that USV duration was a more sensitive measure than USV number of pup stress levels. Once again, there was a dissociation between sedation and USV duration. This was the only manipulation which had opposing effects on USVs and sedation. The cold increased USV rate while impairing performance on the sedation measures, therefore providing strong evidence that the reduction in USV duration is not a by-product of sedation.

Although not tested here, it would be interesting to assess the sensitivity of the duration measure to pharmacologically-induced increases in anxiety. The inverse benzodiazepine agonist FG-7142, for example, which has been shown to induce a stress response in rats (Pellow and File, 1985) does not increase USV number in isolated rat pups (Olivier et al., 1998a,b). The results of the current studies suggest that measuring USV duration may reveal increases in anxiety-like behavior that counting the calls does not reveal. Further tests with anxiogenic pharmacological agents are required to test this hypothesis.

Collectively, these studies provide evidence that USV duration is a measure that is more sensitive to the anxiolytic effects of pharmacotherapies and is easier to dissociate from sedation than the more traditional measure, USV number. This suggests that longer USVs are affectively different than shorter ones. It is possible that a more sophisticated analysis of USVs will produce a measure that is even more sensitive than duration (e.g., USV amplitude) or that a combination of measures will provide a more sensitive means of assessing pup anxiety. Improving assays in this way improves their utility in the search for novel anxiolytics from novel drug classes. Moreover, by providing a measure that is further separated from the sedative effects that accompany higher doses of most anxiolytics will make it easier to dissociate anxiolysis from sedation.

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