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Research article

CRF_1 but not glucocorticoid receptor antagonists reduce separation-induced distress vocalizations in guinea pig pups and CRF overexpressing mouse pups. A combination study with paroxetine☆,☆☆

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ABSTRACT

Rationale: Given the large number of patients that does not respond sufficiently to currently available treatment for anxiety disorders, there is a need for improved treatment.
Objectives: We evaluated the anxiolytic effects of corticotropin releasing factor $(CRF)_1$ receptor antagonists and glucocorticoid receptor (GR) antagonists in the separation-induced vocalization test in guinea pigs and transgenic mice with central CRF overexpression. Furthermore, we explored effects of these drugs when given in combination with a suboptimal dose of a selective serotonin re-uptake inhibitor (SSRI).
Methods: In guinea pig pups, the CRF_1 receptor antagonists CP-154,526 and DMP695, and the GR antagonists mifepristone and Org34517 (all at 2.5, 10 and 40 mg/kg intraperitoneally (IP)) were tested alone or in combination with 0.63 mg/kg paroxetine IP. In CRF overexpressing mouse pups and wild type littermates, effects of CP-154,526 (10, 20 and 40 mg/kg subcutaneously (SC)) and mifepristone (5, 15, 45 mg/kg SC) were studied alone or in combination with 0.03 mg/kg paroxetine SC.
Results: CRF_1 but not GR antagonists reduced the number of calls relative to vehicle in guinea pigs and mice, independent of genotype. Treatment of CRF_1 receptor or GR antagonists with paroxetine had no combined effect in guinea pigs, wild type or CRF overexpressing mice.
Conclusions: Current results indicate robust anxiolytic properties of CRF_1 receptor antagonists in guinea pigs and mice overexpressing CRF, and lack thereof of GR antagonists. Although no combined treatment effects were observed, it would be interesting to study combined treatment of CRF_1 receptor antagonists with SSRIs following chronic drug administration.

1. Introduction

Serotonin re-uptake inhibitors (SSRIs) are among the most frequently described anxiolytics (Baldwin et al., 2014). However, problems encountered with the existing anxiolytics include the large number of non-responders to drug treatment, the delayed onset of action and adverse effects (Stewart et al., 2015). Therefore, there is a need for more effective pharmacotherapy. Such improved pharmacological treatment may be achieved by multi-target drug strategies (Hendriksen and Groenink, 2015; Millan, 2006).

Anxiety disorders are complex disorders as many factors are involved in their pathogenesis. Persistent changes in corticotropin-releasing factor (CRF), a neuropeptide with central and peripheral effects on anxiety, may contribute to the development of anxiety disorders

Abbrevations: CRF, corticotropin-releasing factor; CRFtg, CRF transgenic; DEC-ABC, Animal Ethical Committee of the Academic Biomedical Centre Utrecht; GR, glucocorticoid receptor; HPA axis, hypothalamus-pituitary-adrenal axis; PND, postnatal day; SSRI, serotonin re-uptake inhibitor; USV, ultrasonic vocalization.

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(Heim and Nemeroff, 1999) and is known to play a role in anxiety-like behavior in both humans and rodents (Bale, 2005). CRF_1 receptors are found throughout the brain, including the cerebral cortex, extended amygdala, septum, and brainstem, but in high density in the pituitary (Schmidt et al., 2010; Van Pett et al., 2000). CRF_1 receptors in the pituitary regulate the hypothalamus-pituitary-adrenal (HPA) axis. Activation of the HPA axis by CRF triggers glucocorticoid secretion (e.g. cortisol, corticosterone). In the central nervous system, corticosterone exerts its effect among others through lower-affinity glucocorticoid receptors (GRs). GRs are widely expressed in the hypothalamus and areas that regulate emotions including prefrontal cortex, basolateral amygdala, and hippocampus (Alt et al., 2010; Joëls and de Kloet, 1994).

Considering the role of CRF in the regulation of anxiety, anxiolytic activity of CRF_1 receptor antagonists has been studied and effects are observed depending on test, species and compound under study (Kehne and Cain, 2010). For instance, the non-peptidergic CRF_1 receptor antagonists CP-154,526 and DMP695 both revealed anxiolytic effects in a lick suppression and social interaction test, but not in an elevated plus maze and conditioned vocalization test in adult rats (Millan et al., 2001). Anxiolytic effects of GR antagonists have been less well studied, and reported effects also depend on the specific test, species and compound under study (Korte et al., 1996; Loi et al., 2015; Pugh et al., 1997). In this respect, the GR antagonist mifepristone showed anxiolytic effects in the rat elevated plus maze test immediately after the exposure to a conditioned stressor, but not in the fear-motivated immobility test (Korte et al., 1995). Another potentially interesting GR antagonist is Org34517. Org34517 is a weaker but more selective GR antagonist than mifepristone. In contrast to mifepristone, Org34517, only has limited affinity towards progesterone receptors (Peeters et al., 2008; Sitruk-Ware and Spitz, 2003) and behaves as a full GR antagonist without partial agonist activity (Havel et al., 1996; Peeters et al., 2008). Although to our knowledge Org34517 has not been studied in anxiety tests, Org34517 and mifepristone both attenuated anxiety associated with ethanol withdrawal in rats (Reynolds et al., 2015; Sharrett-Field et al., 2013). A pilot study with 60^+ humans diagnosed with anxiety disorder observed improvements in worry severity, memory and executive function in individuals with higher baseline levels of cortisol after 3–4 weeks of treatment with mifepristone (Lenze et al., 2014). To date, early clinical trial results of both CRF_1 receptor and GR antagonists have been disappointing (Schatzberg, 2015; Stewart et al., 2015), but more extensive studies are required to determine potentially beneficial effects in anxiety patients with HPA axis dysregulation.

Interestingly, several lines of research have shown interactions between CRF and serotonin in the regulation of anxiety-like responses (Lukkes et al., 2009; Meloni et al., 2008). As such, combined treatment of (low dose) SSRIs with CRF_1 receptor antagonists could be beneficial in the treatment of anxiety disorders. For example, CP-154,526 abolished fear acquisition deficits in serotonin transporter knockout rats (Bijlsma et al., 2015). In addition, a study of Heitland et al. (2016) showed an interaction between genetic variants of the CRF_1 receptor and serotonin transporter with regard to human fear acquisition deficits. Combined administration of SSRIs with GR antagonists may offer another potentially beneficial approach for the treatment of anxiety disorders. For instance, corticosterone may modulate emotional behavior indirectly by altering serotonergic neurotransmission (Judge et al., 2004; Linthorst and Reul, 2008). Microdialysis studies in rats support the potentially beneficial effects of combined treatment of SSRIs with GR antagonists. Combined chronic treatment with the GR antagonist Org34850 enhanced the ability of the SSRI fluoxetine to elevate forebrain serotonin levels (Johnson et al., 2007). A subsequent study showed that this effect could be attributed to downregulation of serotonin transporters (Johnson et al., 2009). However, the behavioral effect of CRF_1 receptor antagonists or GR antagonists in combination with SSRIs has not yet been tested in animal models for anxiety.

Here we used the separation-induced distress vocalization test in guinea pigs and mice to determine anxiolytic effects of the treatment conditions of interest. Separation-induced distress vocalization occurs in a wide variety of species, including man. It is an innate emotional response exhibited after a short period of separation from parents, in particular the mother (Pettijohn, 1979). A recent meta-analysis showed that in guinea pigs the test detects a wide range of drug classes in use for the treatment of anxiety disorders, including SSRIs and other antidepressants, making it a valuable screen to study compounds with anxiolytic potential (Groenink et al., 2015).

In light of the above, we determined the effects of the selective CRF_1 receptor antagonists, CP-154,526 and DMP695, and the GR antagonists, mifepristone (i.e. RU486, RU38486) and Org34517, on distress vocalizations in guinea pig pups and in transgenic mouse pups overexpressing central CRF (CRFtg; Dirks et al., 2002a). These transgenic mice have elevated central CRF levels, elevated basal plasma corticosterone levels and a dysregulated HPA axis (Groenink et al., 2002), reminiscent of changes observed in certain human anxiety disorders (Arborelius et al., 1999), and were used to model anxiety disorder with HPA axis dysregulation. In addition, we explored the effects of combined administration of these antagonists with a suboptimal dose of the SSRI paroxetine. We chose to test a suboptimal dose of paroxetine since we expected synergistic effects of this multi-target treatment. Also, in a clinical setting the advantage of a multi-target strategy would be to achieve treatment efficacy by combining lower doses of drugs, which would meanwhile reduce incidence of side effects and circumvent initial anxiogenic effects of SSRIs (Hendriksen and Groenink, 2015; Millan, 2006).

2. Materials and methods

2.1. Animals, breeding and housing conditions

2.1.1. Guinea pigs

Female guinea pigs (*Cavia porcellus*, HsdPoc:DH) were obtained six weeks pregnant (Harlan Laboratories, Venlo, The Netherlands). Eight weeks after arrival, pups were weaned and female dams were re-used for in-house breeding with male guinea pigs (HsdPoc:DH, Harlan Laboratories, Venlo, The Netherlands). All experiments were performed with pups from in-house breeding, except for the Org34517 experiments which were performed with pups from pregnant dams at arrival. Both male and female pups were used for the vocalization experiments.

All pregnant females were maintained on a 12 h/12 h light/dark cycle (lights on from 7:00 AM–7:00 PM), an ambient temperature of 21 ± 1 °C and relative humidity (40–60%). Female guinea pigs were housed in pairs in standard guinea pig cages ($725 \times 620 \times 300$ mm, base surface 4200 cm^2) provided with hay, a shelter and food (Guinea Pig FDI, SDS Diets, UK) and bottled tap water ad libitum. Female pairs remained in these cages throughout gestation and lactation. Each female gave birth to 1–7 (typically 3–5) pups. Occasionally, pups did not survive birth, likely because their body weight was either too low (underdevelopment/too many pups) or too high (asphyxiation during slow birth). A total of 24 breeding pairs and 151 pups were used for the vocalization experiments. Body weight of guinea pig pups, the day before their first vocalization test, ranged from 101 g–264 g. The Animal Ethical Committee of the Academic Biomedical Centre Utrecht (DEC-ABC) approved of all experiments prior to the onset of the study under permit number 2007.I.11.28.

2.1.2. CRFtg mice

Transgenic mice overexpressing neural CRF were generated as described previously (Dirks et al., 2002b). Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF cDNA (0.6-kb fragment), which was inserted into a 8.2-kb genomic DNA-fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence. The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons. Subsequent breeding at the local breeding facilities (Utrecht, The Netherlands) consisted of mating between heterozygous transgenic males

(C57BL/6J background) and C57BL/6JIco females (Charles River, The Netherlands).

In order to obtain pups for the experiments, two female mice were housed per cage (Macrolon type III). CRFtg males were introduced to induce pregnancy (one male and two females). Sires, dams and pups remained in the cages throughout the period of gestation and lactation. Each female gave birth to 1–10 (typically 4–6) pups. A total of 59 breeding couples (two females and one male) and 527 pups (male and female) were used for the vocalization experiments. On vocalization test days, body weight of mouse pups ranged from 2.9 g–8.4 g.

Mice had free access to food-pellets (Standard Rat and Mice Food, SDS Diets, UK) and bottled tap water. Breeding cages had a bedding of woodchips and were enriched with PVC tubes (diameter 5 cm, length 15 cm), Envirodry® (extra bedding) and a disposable mouse home (Bioservices BV, The Netherlands). All mice were housed under a 12-h light/12-dark cycle (lights on from 6:00 AM–6:00 PM) at controlled room temperature (20 ± 2 °C) and relative humidity (40–60%). The experiments were carried out with approval of the DEC-ABC under permit number 2007.I.05.074.

All animals were maintained under specified pathogen-free conditions. Both guinea pigs and mice were housed in the same animal facility (building), except for the guinea pigs used in the experiment with Org34517, which was performed in another building. Background music in all animal rooms was played 24 h/day.

2.2. Drugs

2.2.1. Guinea pigs

Dose-response curves were made for the following drugs: CRF_1 receptor antagonists CP-154,526 and DMP695 and the GR antagonists mifepristone and Org34517. CP-154,526 and DMP695 were dissolved in 2% Tween-80® in saline. Mifepristone and Org34517 were suspended in gelatine-mannitol 0.5%/5% in water. All four test compounds were tested in a dose range of 2.5, 10 and 40 mg/kg. These doses were selected based on tests with these compounds in previous studies with guinea pig pups (Hodgson et al., 2007) and their relative potency (Millan et al., 2001; Groenink et al., 2008). Drugs were given either alone or co-administered with paroxetine hydrochloride hemi-hydrate (0.63 mg/kg, dissolved in saline). This suboptimal dose of paroxetine was chosen based on pilot studies. Paroxetine, tested at 0.63 and 2.5 mg/kg intraperitoneally (IP) with n = 11 per dose, significantly reduced the number of distress calls ($F[2, 30] = 7.1$, $p = 0.003$) at 2.5 mg/kg (Supplementary Fig. 1A; Supplementary Table 3).

All drugs and vehicle were administered IP in a volume of 2 mL/kg. In the co-administration experiments, pups received two injections simultaneously, one on each side of the abdominal midline. The drug solutions were prepared freshly on the morning of each test day. If two or more dosages of the same drug were tested on the same day, the highest dose was diluted to the lower doses. Pups were weighed the day before the test day so that stress effects (separation/weighing) would not affect vocalization behavior on the test day.

2.2.2. CRFtg mice

CP-154,526 (10, 20 and 40 mg/kg, dissolved in 2% Tween-80® in saline) and mifepristone (5, 15, 45 mg/kg, suspended in gelatine-mannitol 0,5%/5% in water) were tested in CRFtg and wild type mice. These doses were chosen based on previous behavioral studies with adult CRFtg mice (Groenink et al., 2008). Drugs were given either alone or co-administered with paroxetine hydrochloride hemi-hydrate (0.03 mg/kg, dissolved in saline). This suboptimal dose of paroxetine was deduced based on pilot studies. Paroxetine, tested in C57BL/6J wild type mice at 0.1, 0.3, 1 and 3 mg/kg subcutaneously (SC) with n = 16–21 per dose, significantly reduced the number of distress calls ($F[4,81] = 8.2$, $p < 0.001$) at all doses (Supplementary Fig. 1B; Supplementary Table 3).

All drugs and vehicle were injected SC (mouse pups were too small for IP injections because of the risk of injecting into the intestines). In the co-administration experiments, the pups received two injections simultaneously, 30 min before testing. Drugs were prepared freshly every day and injected in a volume of 10 mL/kg.

2.2.3. Drugs

CP-154,526 and DMP695 were gifts from Servier, Croissy/Seine France. Mifepristone and Org34517 were gifts from NV Organon, Oss, The Netherlands. Paroxetine was obtained from Pharmacy Mediq, Bergen op Zoom, The Netherlands.

2.3. Experimental set-up

2.3.1. Guinea pigs

Pups were placed in a grey plastic circular arena (diameter 45 cm; height 30 cm). The arena was placed within a big box (80 × 80 × 100 cm). Inside, a white light bulb provided strong illumination. A microphone (IMG stage-line) was placed just above the arena and connected to a pre-amplifier (set at 70 dB) that was connected to an audio filter, which was in turn connected to a PC. Calls were automatically analyzed using the software program Ultravox 2.0 (Noldus, Wageningen, The Netherlands), and the number of calls was quantified. Calls shorter than 15 ms were discarded and calls separated by <15 ms were counted as one call.

2.3.2. CRFtg mice

Two identical experimental set-ups were used. Each of the two ultrasonic pup vocalization devices consisted of a RVS plate (diameter 17 cm), kept at a temperature of 19 °C by circulating water through a reservoir in the plate. A transparent Plexiglas cylinder (diameter 18 cm, height 25 cm) was placed on top of the plate and covered with a Plexiglas top on which a microphone was mounted. For the CP-154,526 dose-response curve, ultrasounds were recorded and analyzed with Ultravox. The microphone (SM2, Ultra Sound Advice, UK) was connected to a bat detector (S-25, Ultra Sound Advice, UK), which was set at 80 kHz. The detector was connected to an audiofilter (Noldus Inc), which translated the analogue signals into digital block pulses. These block pulses were consequently sent to a Pentium 4 computer running Ultravox 2.0 (Noldus Inc., Wageningen, The Netherlands). Onset and end of each call were timed to the nearest ms and total number of ultrasounds produced by each individual animal was calculated. Ultrasounds of all other mice (CP-154,526 + paroxetine and mifepristone studies) were recorded and analyzed with Sonotrack™ (Metris B.V. Hoofddorp, version 1.4.0, product code ST-C004). Sonotrack™ measured ultrasonic vocalization (20–100 kHz) automatically for 5 min (signal level = 23 dB and peak width = 4 ms).

2.4. Experimental procedure

2.4.1. Guinea pigs

On postnatal day (PND) 7 pups were marked and underwent a 5 min pre-experimental screen test. Individuals that spent >30 s vocalizing were included in subsequent drug experiments. Pups spending <30 s vocalizing underwent a second and, if necessary, a third screen test the following days. Pups not reaching the criterion in any of the screen tests were excluded from the drug experiment (approximately 7%).

Each test day followed the same paradigm: a selected pup (PND8-PND30) was injected IP carefully and quickly with the drug(s) above the home cage, and immediately returned to its home cage. Thirty min after the injection(s), the pup was gently removed from the home cage and, in an adjacent room, immediately placed in the centre of the test arena. After 5 min of recording, the pup was removed from the arena and immediately placed back in the home cage. During the test, there were no experimenters present in the test room.

Each pup received three doses of one drug plus one (vehicle) or two (vehicle and vehicle-paroxetine) vehicle treatments according to a Latin

square design with three or four washout days between each dosing to prevent carry-over effects of the injected drugs.

2.4.2. CRFtg mice

On the test day, mouse pups (PND7-PND11) were separated from their mother by removing groups of 4 pups and a handful of bedding material from the home cage. Pups were kept together in a separation cage that was placed on a heating pad to maintain nest temperature. Pups were weighed, sexed, injected and then marked with a marker. Pups were injected SC with vehicle or one of the drug doses or with vehicle and paroxetine or paroxetine and one of the drug doses and put back into the separation cage. Thirty min later each pup was placed singly on one of the RVS plates. Animals remained on the plate for 5 min, while their ultrasonic vocalizations (USVs) were automatically registered. During the test, experimenters were present in the room.

After these measurements, an ear punch was taken for genotyping the pups and pups were returned to their home cages. Material for genotyping was stored till after the experiments. Since genotyping of the mice could only be performed after running the vocalization tests, this resulted in unequal group sizes.

Following the procedure previously described by Dirks et al. (2002a), ear DNA of the offspring was extracted with a High Pure polymerase chain reaction (PCR) Template Preparation Kit (Boehringer, Mannheim, Germany) and screened using PCR with transgene-specific primers. The forward-primers were specific for rat CRH and the reversed-primers originated from the Thy-1 promotor, thus excluding the possibility that the endogenous CRH and Thy-1 gene were amplified.

Each mouse pup was tested only once. Since multiple pups from one litter were used, siblings were allocated a treatment (vehicle, vehicle-paroxetine or a drug dose) via a randomized block design to ensure that litters were represented in a maximum possible number of treatments, and conditions were equally divided over the days. Testing of guinea pigs (between 8.30 AM–1.00 PM (when tested in the second phase of the inactive period, guinea pig pups hardly vocalized) and mice (between 8:30 AM–16:30 PM) was done during the animals' inactive period. After every 5 min session, the test environment was thoroughly cleaned with water and soap and then dried. Experimenters were blinded to genotype, but not to the experimental treatment conditions.

2.5. Statistical analyses

A priori power calculation with effect size 1.1, alpha of 0.05, and a power of 0.8, indicated a group size of 12 guinea pig pups was needed to reliably detect a treatment effect (Gpower http://www.gpower.hhu.de/en.html). Calculation of the effect size was based on mean and variance values from our previous work (Brocco et al., 2008). For CRFtg pups, a priori power calculation with an effect size of 1.2, alpha of 0.05, and a power of 0.8, indicated a total number of 12 was needed to detect a reliable treatment effect (Gpower, http://www.gpower.hhu.de/en.html). The effect size of interest was determined with GPower, using mean and variance values based on our previous work (Vinkers et al., 2010).

A single pup was considered as the unit of analysis. Since sex did not have an effect on the results in either guinea pigs or CRFtg mice, data of male and female pups were pooled. For the guinea pig studies one-way ANOVAs were used to analyze drug effects on the number of calls emitted during the 5 min separation period. Dunnett's post hoc tests were used to assess the statistical significance of individual doses versus vehicle in the dose-response studies, or versus vehicle-paroxetine treatment in the co-administration studies. In addition, the effect of vehicle-paroxetine treatment relative to vehicle-vehicle treatment was determined using independent Student's *t*-tests to confirm the suboptimal dose of paroxetine alone did not already have an effect on vocalization behavior. Significance was defined as $p < 0.05$.

For the CRFtg mice the dose-response study of CP-154,526 and combined administration with paroxetine were both analyzed using a two-way ANOVA, with treatment and genotype as between subjects factors. Simple contrasts were used to determine the effects of individual doses relative to vehicle-treatment. A separate two-way ANOVA was performed to determine the effect of paroxetine in both genotypes. The effects of mifepristone alone or combined with paroxetine were analyzed using a three-way ANOVA, with mifepristone, paroxetine and genotype as between subjects factors. Comparisons between vehicle and different drug dosages were made by simple contrasts.

Outliers (defined as two standard deviations ± mean) were identified with the Boxplot test (SPSS20) and excluded from further analyses. For details on specific outliers in particular treatment groups see Supplementary Table 1.

For detailed information on materials and methods according to the Gold Standard Publication Checklist (Hooijmans et al., 2010) see Supplementary Table 2.

3. Results

An overview of experimental data on number of calls, including number of animals and outliers per experimental group, can be found in Supplementary Table 1.

3.1. Guinea pigs

3.1.1. Dose-response studies of CRF_1 receptor antagonists and GR antagonists

As shown in Fig.1A, CP-154,526 significantly reduced the number of calls ($F[3, 48] = 4.1$, $p = 0.012$). Post hoc analysis showed the number of calls was significantly reduced at 40 mg/kg CP-154,526 compared to vehicle treated guinea pigs. DMP695 significantly reduced the number of calls ($F[3, 46] = 7.4$, $p < 0.001$; Fig. 1C). Post hoc analysis revealed that the number of calls was significantly reduced at 10 and 40 mg/kg DMP695. Treatment with mifepristone or Org34517 did not have a significant effect on the number of calls ($F[3, 44] = 0.8$, $p = 0.48$; Fig. 1E, and ($F[3, 36] = 0.1$, $p = 0.9$; Fig. 1G respectively).

3.1.2. CRF_1 receptor and GR antagonists combined with paroxetine

As displayed in Fig. 1B, co-administration of CP-154,526 and paroxetine (0.63 mg/kg) significantly reduced the number of calls ($F[3, 52] = 8.7$, $p < 0.001$). Post hoc analysis showed that co-administration of paroxetine with either 10 or 40 mg/kg CP-154,526 significantly reduced the number of calls compared to vehicle-paroxetine treatment. Combined treatment of DMP695 with paroxetine (0.63 mg/kg) did not have a significant effect on the number of calls ($F[3, 43] = 2.1$, $p = 0.11$; Fig. 1D). Co-administration of either mifepristone or Org34517 with paroxetine (0.63 mg/kg) did not have a significant effect on the number of calls (treatment effect mifepristone + paroxetine: $F[3, 42] = 0.3$, $p = 0.8$; Fig. 1F and treatment effect Org34517 + paroxetine: $F[3, 50] = 0.1$, $p = 0.5$; Fig. 1H).

3.1.3. Effects of suboptimal dose of paroxetine

Separate *t*-tests, comparing vehicle-vehicle and vehicle-paroxetine treated guinea pigs showed that paroxetine (0.63 mg/kg) when given alone had no significant effect on the number of calls in any of the four co-administration studies ($p < 0.05$; Fig. 1).

3.2. CRFtg mice

3.2.1. CP-154,526 dose-response curve and combined treatment with paroxetine

The effects of CP-154,526 alone and combined with paroxetine were studied in two separate cohorts of mice. In both cohorts, CRFtg mice emitted more USVs than wild type mice (main effect genotype for

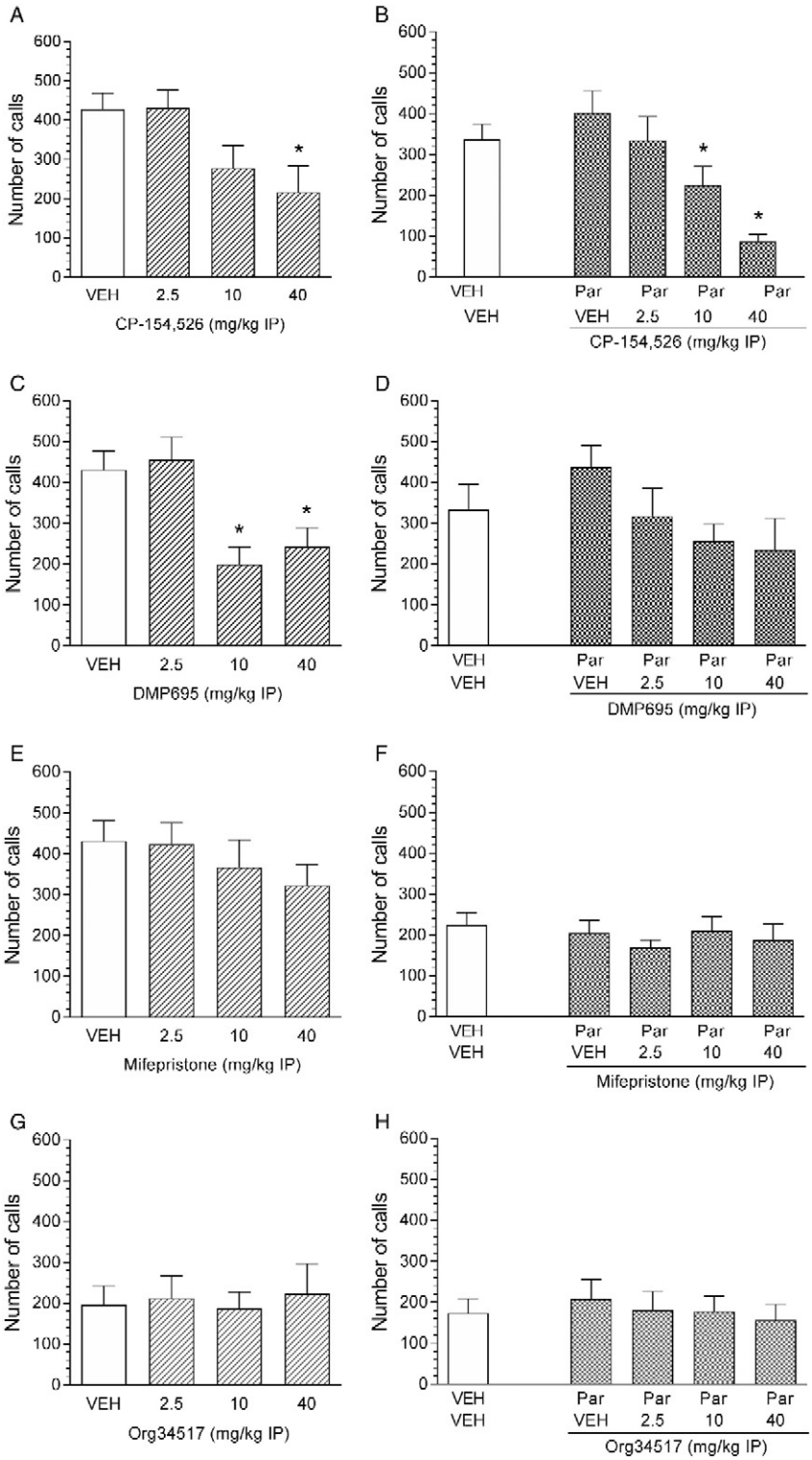

Fig. 1. Effects of CRF_1 receptor and GR antagonists on the number of calls in guinea pigs, as dose-response curves (A, C, E, G) or co-administered with 0.63 mg/kg paroxetine (B, D, F, H). CP-154,526 (A, B), DMP695 (C, D), mifepristone (E, F), Org34517 (G, H). Bars represent mean ± SEM with group sizes: (A) n = 13; (B) n = 12–15; (C) n = 12–13; (D) n = 10–13; (E) n = 12 (F) n = 11–12; (G) n = 9–11; (H) n = 12–15. For details on the number of animals and outliers per treatment condition see Supplementary Table 1. IP, intraperitoneally; Par, paroxetine; VEH, vehicle. * significant difference ($p < 0.05$) from vehicle (A, C, E, G) or vehicle-paroxetine (B, D, F, H).

dose-response study $F[1, 25] = 6.35$, $p = 0.013$; Fig. 2A; for combination study $F[1, 122] = 7.5$, $p = 0.007$; Fig. 2B).

As shown in Fig. 2A, CP-154,526 had a significant effect on the total number of USVs ($F[3, 125] = 8.1$, $p < 0.001$), which was independent of genotype (genotype x dose: $F[3, 125] = 2.1$, $p = 0.11$). Simple contrasts showed that treatment with 10 and 20 mg/kg CP-154,526 significantly reduced the total number of USVs. Combined treatment with CP-154,526 and paroxetine (0.03 mg/kg) had no effect on the total number

of USVs in CRFtg and wild type mice (main effect combined treatment: $F[3, 122] = 2.0$, $p = 0.12$; interaction effect genotype x treatment: $F[3, 122] = 1.35$, $p = 0.26$; Fig. 2B).

3.2.2. Effects of suboptimal dose of paroxetine

Separate analysis comparing vehicle-paroxetine and vehicle-vehicle treated mice showed that paroxetine (0.03 mg/kg) had no effect on the number of USV in any of the genotypes (main effect paroxetine: $F[1, 74] = 1$, $p = 0.3$; paroxetine x genotype: $F[1, 74] = 0.9$, $p = 0.8$; see first two sets of bars in Fig. 2B).

3.2.3. Mifepristone dose-response curve and combined treatment with paroxetine

The effects of mifepristone on USVs in CRFtg mice with or without addition of paroxetine were studied within one experiment and analyzed with a 3-way ANOVA (with genotype, paroxetine and mifepristone as between subjects factors). Similar to the CP-154,526 studies, CRFtg mice emitted significantly more USVs than wild type mice (main effect genotype: $F[1, 199] = 15.4$, $p < 0.001$).

Mifepristone had a significant effect on the number of USVs ($F[3, 199] = 3.6$, $p = 0.014$; Fig. 3). This effect was independent of genotype ($F[3, 199] = 1.8$, $p = 0.5$) and could not be attributed to a particular dose in the post hoc analysis. The effect of mifepristone was independent of administration with 0.03 mg/kg paroxetine ($F[3, 199] = 1.8$, $p = 0.15$), and the combined treatment effect did not depend on genotype (genotype x mifepristone x paroxetine: $F[3, 199] = 0.6$, $p = 0.6$).

3.2.4. Effects of suboptimal dose of paroxetine

Paroxetine (0.03 mg/kg) had no effect on the number of USVs (main effect paroxetine: $F[1, 199] = 2.8$, $p = 0.1$; paroxetine x genotype: $F[1, 199] = 1.6$, $p = 0.2$; Fig. 3).

4. Discussion

In the present study we found that CRF_1 receptor antagonists, but not GR antagonists, showed anxiolytic-like effects in separation-induced distress vocalization tests in guinea pig pups and transgenic mouse pups overexpressing central CRF. We further showed that combined treatment of CRF_1 receptor or GR antagonists with a suboptimal dose of paroxetine had no additional effect.

4.1. CRF_1 receptor antagonists

The CRF_1 receptor antagonists CP-154,526 and DMP695 both reduced the number of distress calls across dose-ranges similar to those required for actions at CRF_1 receptors in the brain (Gannon and Millan, 2006; Griebel et al., 1998; Kehne et al., 2000; Lorrain et al., 2005; Millan et al., 2001). For CP-154,526 similar effects on separation-induced distress calls have been reported in guinea pig pups (Hodgson et al., 2007) and rat pups (Hodgson et al., 2007; Iijima and Chaki, 2005; Kehne et al., 2000), although CP-154,526 did not block conditioned USV in adult rats (Millan et al., 2001). In these studies it was also shown that CP-154,526 reduced the number of vocalizations at dose ranges (1–40 mg/kg) that were without effect on negative geotaxis, righting reflex or body temperature (Hodgson et al., 2007; Iijima and Chaki, 2005; Kehne et al., 2000). Although we did not control for potential drug-induced side effects that could affect vocalization behavior in the present study, it seems unlikely that the anxiolytic-like effects of CP-154,526 and DMP695 were due to such side effects since we tested these drugs in a similar dose range. Furthermore, in CRFtg mice and their corresponding wild types, neither CP-154,526 or DMP695 (or mifepristone or Org34517) reduced the acoustic baseline startle response in the dose range tested in the current study (Groenink et al., 2008). These findings further strengthen the idea that the reduction in calls found in the present study are not due to sedative or muscle-reflexive drug effects. We are the first to test DMP695 in the guinea pig vocalization test, and found an anxiolytic-like effect of DMP695. Since both CRF_1 receptor antagonists show high selectivity for CRF_1 versus CRF_2 and other sites (Gilligan and Li, 2004; Gilligan et al., 2000; Lejeune and Millan, 2003; Millan et al., 2001; Schulz et al., 1996), these findings confirm and extend the activity of different non-peptidergic CRF_1 receptor antagonists (SSR125543A, NBI27914) in the maternal separation-induced vocalization tests (Griebel et al., 2002; Ise et al., 2008). Effects of CRF_1 receptor antagonists in other anxiety tests appear less consistent (Kehne and Cain, 2010, for review). This may be explained by the fact that beneficial effects of CRF_1 receptor antagonists in anxiety tests likely depend on sufficiently high stress levels and considerable involvement of CRF neuronal circuitry in modulation of the response under study (de Jongh et al., 2003; Kehne and Cain, 2010; Valdez, 2006).

To our knowledge, this is the first time anxiolytic effects of CRF_1 receptor antagonists have been tested in an animal model with HPA axis dysregulation. We studied the effects of CP-154,526 on separation induced ultrasonic distress vocalization in CRFtg mice. Firstly, the study showed that transgenic mice overexpressing CRF emitted more USVs than wild type mice when separated from their mother. This result is in line with earlier findings that these and other transgenic mice overexpressing CRF display an exaggerated response to stress, which becomes particularly evident in more stressful situations (Groenink et al., 2003; Stenzel-Poore et al., 1994). According to a telemetric study, CRFtg mice show a moderate elevation in basal body temperature

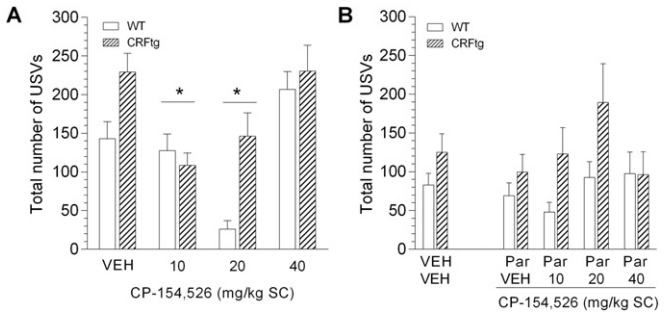

Fig. 2. Effects of CP-154,526 on the number of ultrasonic vocalizations (USVs) in wild type and CRFtg mice, as a dose-response curve (A) or co-administered with 0.03 mg/kg paroxetine (B). Bars represent mean ± SEM with group sizes: (A) WT: n = 9–22, CRFtg: n = 8–28; (B) WT: n = 15–25, CRFtg: n = 12–16. For details on the number of animals and outliers per treatment condition see Supplementary Table 1. WT, wild type; CRFtg, CRF transgenic; SC, subcutaneously; Par, paroxetine; VEH, vehicle. (A) * significant difference ($p < 0.05$) from vehicle, following main effect of CP-154,526.

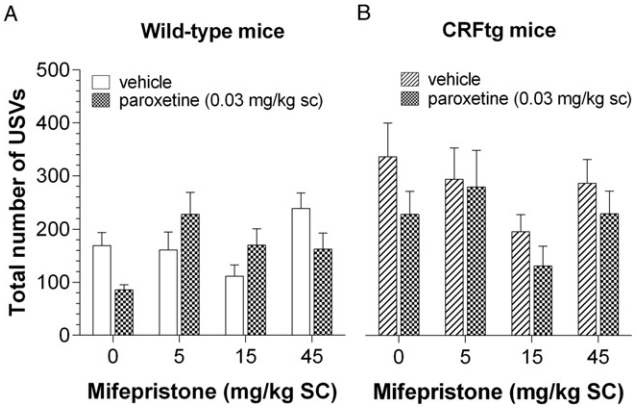

Fig. 3. Effects of mifepristone on the number of ultrasonic vocalizations (USVs), as a dose-response curve or co-administered with 0.03 mg/kg paroxetine in wild type (A) and CRFtg (B) mice. Bars represent mean ± SEM with group sizes: (A) vehicle: n = 10–17, paroxetine: n = 8–17; (B) vehicle: n = 10–19, paroxetine: n = 10–15. Mifepristone had a significant main effect on the number of USVs ($F[3, 199] = 3.6$, $p = 0.014$. For details on the number of animals and outliers per treatment condition see Supplementary Table 1. CRFtg, CRF transgenic; SC, subcutaneously.

relative to wild type mice during the second half of the inactive period (Dirks et al., 2002a). This time constraint genotype difference in core body temperature is not present upon handling and following injection (Vinkers et al., 2012), and the decrease in body temperature following exposure to the test plate is similar for both genotypes (unpublished data). Therefore, the increase in distress vocalizations observed in CRFtg mice is probably not related to differences in body temperature.

In addition, we showed that CP-154,526 at lower doses significantly reduced the number of USVs in wild type and CRFtg mice. This in an important finding since it indicates that CRF_1 receptor antagonists can also exert anxiolytic-like effects under conditions of chronic stress. The observed U-shaped dose-response curve of CP-154,526 in mice was unexpected, as this has not previously been reported in separation-induced vocalization tests using guinea pigs and rats (Hodgson et al., 2007; Iijima and Chaki, 2005; Kehne et al., 2000). Studies on the effects of CRF administration on vocalization behavior in pups indicate that both the magnitude and direction of effects are dependent on stress level (plate temperature), which appears different for rats and mice (Dirks et al., 2002c; Harvey and Hennessy, 1995; Ise et al., 2008). As such, the observed U-shaped dose-response of effect of CP-154,526 may be explained by the delicate balance between endogenous CRF levels, CRF_1 receptor activation and associated behavioral strategies (Dirks et al., 2002c). Alternatively, CP-154,526 has been reported to exert weak partial agonist actions at CRF_1 receptors (Grosjean-Piot et al., 1997). As such, an inverted U-shape dose-response curve in rat social interaction (Millan et al., 2001) and submaximal reduction of CRF-induced effects have been reported occasionally (Schulz et al., 1996).

In our previous work, we demonstrated that these CRF overexpressing mice have elevated baseline corticosterone levels and reduced GR function (Groenink et al., 2002). In addition, we showed that CRF_1 receptor antagonists decrease plasma corticosterone levels in both wild type and CRF transgenic mice, while GR antagonists increase corticosterone levels in wild type but not in CRF transgenic mice (Groenink et al., 2008). Together with the current finding that blockade of CRF_1 receptors, but not GRs, reduces vocalizations in CRF overexpressing and wild type mice, this would imply that centrally located CFR_1 receptors are involved in the modulation of distress vocalizations by CRF_1 receptor antagonists, and that involvement of corticosterone herein is unlikely. Yet, it cannot be excluded that the corticosterone reducing effects of CP-154,526 and DMP695 contribute to their anxiolytic effects. CRF_1 receptor antagonists and GR antagonists are likely to differentially affect the balance between mineralocorticoid receptor and GR activation. This balance may be particularly important in the regulation of anxiety behavior (Korte, 2001; Roozendaal et al., 1996).

4.2. GR antagonists

Mifepristone and Org34517 did not alter separation-induced vocalizations in guinea pigs, wild type mice or CRF overexpressing mice. Absence of effects for mifepristone in the fear-potentiated startle and defense burying paradigm in rats has also been reported (Korte et al., 1996). To our knowledge, studying the anxiolytic-like properties of Org34517 is a novelty. Studies that did report anxiolytic effects of mifepristone, used tests that were either based on fear learning and conditioning (Korte et al., 1995; Pitman et al., 2011; Pugh et al., 1997) or determined effects of mifepristone after early life stress or acute pre-exposure to a stress factor (Jakovcevski et al., 2011; Loi et al., 2015). The latter suggests that GR antagonists may exert anxiolytic-like effects only in subjects with compromised HPA axis activity. The absence of effect of mifepristone on vocalization behavior in CRF overexpressing mice in this study however, disproves that. Considering the reported beneficial effects of mifepristone on fear learning and conditioning, it would be interesting to study the effects of GR antagonists in contextual and cue conditioning in adult CRF overexpressing mice.

It cannot be fully excluded that the injection test interval was too short to detect beneficial effects of mifepristone on distress vocalizations in the present study. Still, we previously showed that both mifepristone and Org34517 enhance corticosterone secretion in wild type mice within 30 min after administration (Groenink et al., 2008). However, such acute effects may be related to the short-term, nongenomic effects attributed to glucocorticoids which may occur within minutes (Song and Buttgereit, 2006; Tasker et al., 2006). Glucocorticoids also exert many effects via changes in gene expression, and therefore, effects of GR antagonists may be delayed and long-lasting (Moldow et al., 2005; Servatius et al., 2005). On the other hand, studies with a protein synthesis inhibitor showed that genomic effects of glucocorticoids can already be evident within 20–25 min (Mikics et al., 2004).

4.3. CRF_1 receptor antagonists in combination with paroxetine

Microdialysis studies have shown that changes in CRF_1 receptor pathways alter serotonergic neurotransmission (Linthorst and Reul, 2008), indicating the possibility for interactions between SSRIs and CRF_1 receptor antagonists. The reversal of fear acquisition deficits in serotonin transporter knockout rats by CP-154,526 also suggests functional interactions between CRF and serotonin pathways (Bijlsma et al., 2015). Yet, our data did not indicate such interactions. Although CP-154,526 given in combination with paroxetine may have induced a slight leftward shift relative to the anxiolytic-like effects of CP-154,526 alone in guinea pigs, this combination was without effect on distress calls in CRF transgenic mice. Also DMP695 combined with paroxetine did not reduce the number of calls in guinea pigs. To our knowledge this combination of compounds has not been studied in anxiety tests before. The net result of the interaction between the serotonergic and CRF systems appears complex, as has also been demonstrated in electrophysiology studies. Local application of CRF in the dorsal raphe nucleus results in decreased or increased firing rates of serotonin neurons depending on the dose used and the subset of neurons studied (Kirby et al., 2000; Linthorst and Reul, 2008; Lowry et al., 2000; Lukkes et al., 2009; Price and Lucki, 2001). Under the conditions of the present study, the combination of CRF_1 receptor antagonists and SSRIs apparently does not yield beneficial effects on anxiety behavior.

4.4. Paroxetine in guinea pigs and CRFtg mice

Paroxetine is a well-known effective anxiolytic drug. One of the potential advantages of multi-target therapy is that lower drug doses could be used, thereby reducing side effects (Millan, 2006). We therefore used a suboptimal dose of paroxetine to explore a possible synergistic anxiolytic response of paroxetine combined with CRF_1 receptor or GR antagonists. In pilot vocalization studies we tested paroxetine in a dose-response curve in guinea pigs and in CRFtg mice. These studies indicated that paroxetine in a dose of 0.63 mg/kg IP in guinea pigs and doses lower than 0.1 mg/kg SC in CRFtg mice did not affect the number of calls. Results of the present studies indeed show that these doses were without effect on vocalization behavior.

Although acute treatment with SSRIs may have anxiogenic effects in humans, such effects are not consistently reported following acute dosing in healthy animals. Reported effects are unequivocal and range between anxiolytic (Borsini et al., 2002), no effect (de Jongh et al., 2002), and anxiogenic (Birkett et al., 2011). In our study paroxetine reduced the number of calls in guinea pig pups (Supplementary Fig. 1; Supplementary Table 3), indicative of an anxiolytic-like profile. This is in line with all previously reported effects of SSRIs in this test except for one (Hudzik et al., 2003; for review see Groenink et al., 2015), and makes that vocalization tests are considered good screening tests with high predictive validity (Groenink et al., 2015).

In our study, guinea pig pups were tested repeatedly with a three to four day interval, which is a quite common approach (for review Groenink et al., 2015). The use of a balanced Latin square design in the present study however, makes it unlikely that the test outcome

(number of calls) was affected by developmental, carry over or intermittent dosing effects. The anxiolytic-like effects of SSRIs reported in literature appear independent of the study design used. Both studies using a between subjects design as well as studies using a within subject study design found that acute treatment with SSRIs reduced the number of calls in guinea pig pups (for review see Groenink et al., 2015).

In CRFtg mice, paroxetine also did not have anxiogenic-like effects, but rather reduced the number of calls (Supplementary Fig. 1; Supplementary Table 3). Thus, although central CRF overexpression results in increased distress vocalization upon separation, it does not alter the acute behavioral response to SSRIs.

Finally, in both the guinea pig and mouse tests the basal level of vocalization varies considerably between cohorts of animals. Such variation in the number of calls has also been reported by other groups as detailed in a review by Groenink et al. (2015), and may have been enhanced by differences in breeding and housing in this particular study (see Materials and Methods section).

4.5. GR antagonists in combination with paroxetine

Dysregulated interactions between glucocorticoids and the serotoninergic system can alter susceptibility to anxiety disorders (Kalafatakis et al., 2015). And although promising synergistic neurochemical data of GR antagonists and the SSRI fluoxetine exist (Johnson et al., 2007; Johnson et al., 2009), GR antagonists together with a suboptimal dose of paroxetine did not reveal anxiolytic effects in our vocalization models; not in normal healthy animals, nor in transgenic mice with HPA axis dysregulation. To the best of our knowledge this is the first study to determine effects of combined treatment of an SSRI with GR antagonists on anxiety behavior. Mifepristone did facilitate the anxiolytic effect of low doses of diazepam in a burying test in diabetic mice (López-Rubalcava et al., 2013). These diabetic mice have increased anxiety behavior due to hyperactivation of the HPA axis. That study shows that, despite our negative findings, it may be worthwhile to further explore the potential of combining GR antagonists with anxiolytics.

5. Conclusions and future research

We showed that the CRF_1 receptor antagonist CP-154,526 has anxiolytic effects in a separation-induced distress vocalization test in transgenic mice with central CRF overexpression. In addition, the CRF_1 receptor antagonist DMP695 was tested in the guinea pig vocalization test for the first time and exerted anxiolytic effects. These observations in both guinea pigs and mice with a dysregulated HPA axis, confirm and extend earlier findings regarding anxiolytic properties of CRF_1 receptor antagonists. Although CRF_1 receptor antagonists have not yet shown positive results in clinical trials (Schatzberg, 2015), our transgenic animal data point towards exploration of clinical research in anxiety patients with HPA axis dysregulation.

Further, we are the first to show that GR antagonists are devoid of anxiolytic effects in separation-induced vocalization tests.

Although the results of this study do not indicate that the novel approach of combined treatment of CRF_1 receptor or GR antagonists and SSRIs would have added value over single drug treatment, further studies on combined studies are warranted. An important addition would be to study such combined treatments after chronic drug administration. Unfortunately, this is not possible in our vocalization model since pups only emit calls in a small age window. Further valuable additions would be to study the combination of drugs in a wider dose range and with different SSRIs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pbb.2017.01.003.

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