



Anxiolytic-like effects of the neurokinin 1 receptor antagonist GR-205171 in the elevated plus maze and contextual fear-potentiated startle model of anxiety in gerbils

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Gerbils show a neurokinin (NK)1 receptor pharmacological profile, which is similar to that observed in humans, and thus have become a commonly used species to test efficacy of NK1 receptor antagonists. The aim of this study was to determine whether systemic administration of the NK1 receptor antagonist GR-205171 produced anxiolytic-like effects in the elevated plus maze and in a novel contextual conditioned fear test using fear-potentiated startle (FPS). On the elevated plus maze, treatment with GR-205171 at 0, 0.3, 1.0, and 5.0 mg/kg doses, 30 min before testing produced anxiolytic-like effects in an increasing dose–response manner as measured by the percentage of open arm time and percentage of open arm entries. For contextual fear conditioning, gerbils were given 10 unsignaled footshocks (0.6 mA) at a 2-min variable interstimulus interval in a distinctive training context. Twenty-four hours after training, gerbils received treatment of GR-205171 at 0, 0.3, 1.0, and 5.0 mg/kg doses, 30 min before testing in which startle was elicited in the same context in which they were trained. Contextual FPS was defined as an increase in startle over pretraining baseline values. All drug dose

levels (0.3, 1.0, and 5.0 mg/kg) significantly attenuated contextual FPS when compared with the vehicle control group. A control group, which received testing in a different context, showed little FPS. These findings support other evidence for anxiolytic activity of NK1 receptor antagonists and provide a novel conditioned fear test that may be an appropriate procedure to test other NK1 antagonists for preclinical anxiolytic activity in gerbils. *Behavioural Pharmacology* 00:000–000 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The mammalian tachykinins include substance P (SP), neurokinin (NK)A, and NKB, the effects of which are preferentially mediated by the G protein-coupled receptors NK1, NK2, and NK3, respectively (Regoli *et al.*, 1994; Pennefather *et al.*, 2004). In the nervous system, tachykinins operate as neurotransmitters and neuromodulators, and have historically been implicated in a wide variety of biological actions, including pain transmission, inflammation, smooth muscle contraction, vasodilation, gland secretion, and activation of the immune system (Kramer *et al.*, 1998; Quartara and Maggi, 1998; Severini *et al.*, 2002). More recent evidence suggests that the central SP–NK1 system is also involved in various stress-related pathologies, including anxiety and depression (for review see, Ebner and Singewald, 2006). In mammals, SP is the most abundant tachykinin in the central nervous system, where, along with NK1, it is widely distributed in brain regions involved in the regulation of affective behavior and mediation of stress responses, such as the amygdala, septum, hippocampus,

hypothalamus, and periaqueductal gray (Hokfelt *et al.*, 1985; Maeno *et al.*, 1993; Szeideemann *et al.*, 1995; Barbaresi, 1998; Commons and Valentino, 2002; Hietala *et al.*, 2005; Rigby *et al.*, 2005; Nagano *et al.*, 2006).

Earlier animal studies have shown that exposure to a variety of aversive and stressful situations alter SP transmission in various brain regions (Bannon *et al.*, 1986; Siegel *et al.*, 1987; Rosen *et al.*, 1992; Brodin *et al.*, 1994; Kramer *et al.*, 1998; Ebner *et al.*, 2004). In addition, both systemic and central injections of SP agonists elicit a variety of anxiety-like behaviors in animals, including conditioned place aversion (Elliott, 1988; Aguiar and Brandao, 1994), more time in the closed arms of the elevated plus maze (Aguiar and Brandao, 1996; Teixeira *et al.*, 1996; De Araujo *et al.*, 2001; Duarte *et al.*, 2004; Bassi *et al.*, 2007), and enhanced inhibitory avoidance learning (Pelleymounter *et al.*, 1986; Hasenohrl *et al.*, 1990). In contrast, pharmacological blockade of SP with NK1 receptor antagonists reduced depressant-like and anxiety-like behaviors in different animal species (Kramer

et al., 1998; Rupniak *et al.*, 2001, 2003b; Varty *et al.*, 2002a; Dableh *et al.*, 2005; Woolley *et al.*, 2006). NK1 antagonists also have a robust anxiolytic effect in various conditions of the social interaction test of anxiety (File, 1997; Cheeta *et al.*, 2001; Gentsch *et al.*, 2002).

These findings have led pharmaceutical companies to develop a variety of selective and potent NK1 antagonists to offer new options for the treatment of anxiety and depression (for recent review, see Quartara and Altamura, 2006). Unfortunately, assessing the preclinical efficacy of these NK1 antagonists has been complicated by marked differences in the pharmacology between human and rat or mouse NK1 receptors. Most NK1 receptor antagonists that display high affinity at human receptors show low affinity, selectivity, and brain penetration in rats and mice, because of differences in the amino acid sequence of the NK1 receptor (Beresford *et al.*, 1991; Fong *et al.*, 1992), thus necessitating the administration of high doses that typically results in unspecific pharmacological effects in these rodents (Rupniak and Jackson, 1994; Smith *et al.*, 1994; Rupniak *et al.*, 2001, 2003a). The NK1 receptor antagonist GR-205171 has high binding selectivity for rat NK1 receptors; however, as observed with other NK1 receptor antagonists, the ability of GR-205171 to inhibit SP binding in rat NK1 receptors is low, with binding affinity values (K_i) approximately 40-fold higher than those obtained using human NK1 receptors (Gitter *et al.*, 1991; Saria, 1999). Furthermore, GR-205171 causes only a moderate rightward shift in SP-induced inositol-1-phosphate accumulation and acidification rate in cells expressing rat NK1 receptors relative to human NK1 receptors.

In contrast to mice and rats, gerbils show an NK1 receptor pharmacological profile which is very similar to that observed in humans (Saria, 1999; Griffante *et al.*, 2006), and thus the gerbil has become a commonly used preclinical model to test efficacy of NK1 receptor antagonists. In-vivo studies show that the doses of GR-205171 needed for central NK1 receptor occupancy are several hundred-folds higher for rats than gerbils. For example, in gerbils the concentration needed for 50% inhibition of NK1 agonist-induced foot drumming (ID_{50}) is 0.02 mg/kg; however, in rats, 10–30 mg/kg of GR-205171 are needed to significantly reduce SP-induced sniffing and agonist-induced hypertension (Rupniak *et al.*, 2003a).

In clinical trials, several studies have shown that NK1 antagonists, including GR-205171, show promise as treatment options for individuals suffering with pathological anxiety (Kramer *et al.*, 2003). For example, as a treatment for symptoms of social phobia, the efficacy of GR-205171 is reportedly similar to that of citalopram (Furmark *et al.*, 2005). The NK1 receptor antagonist GW597599 is effective against CO₂-induced panic attack

with an efficacy similar to alprazolam (McLean, 2005). In a more recent study, George *et al.* (2008) have shown that the NK1 receptor antagonist LY686017 suppresses spontaneous alcohol cravings and blunts craving and cortisol responses induced by alcohol-cue challenges in alcohol-dependent subjects with high trait anxiety. Currently, phase II clinical trials are also evaluating the effectiveness of GR-205171 in decreasing symptoms of posttraumatic stress disorder.

At present, relatively few studies have examined the effects of NK1 antagonists on conditioned fear. The fear-potentiated startle (FPS) paradigm may be particularly useful for examining conditioned fear in gerbils, because fear induces foot drumming in this species, which inhibits the expression of conditioned freezing – a behavior used to index levels of fear and anxiety in many conditioning paradigms (Rupniak *et al.*, 2003a; Woolley *et al.*, 2006). Furthermore, because much is known about the neuro-circuits mediating startle and the augmentation of startle by states of fear and anxiety, the FPS paradigm may be particularly useful in future studies designed to identify central mechanisms responsible for anxiolytic-like actions of NK1 antagonists. Thus, the purpose of this study was to determine whether the NK1 receptor antagonist GR-205171 inhibits contextual conditioned fear using the FPS paradigm. As past research has shown that a number of NK1 receptor antagonists produce an anxiolytic-like profile in gerbils as assessed by the elevated plus maze (Varty *et al.*, 2002a), we also examined whether GR-205171 produces anxiolytic-like effects in the elevated plus maze.

Methods

Subjects

Male Mongolian gerbils (Charles River Laboratories International Inc., Wilmington, Massachusetts, USA) weighing between 60 and 75 g at the onset of testing were used in all experiments. Gerbils were housed four per cage with food and water freely available in a room maintained under constant temperature (22°C). Gerbils were allowed 2 weeks to acclimate to the housing conditions before the start of experiments. All testing was performed in the light phase of a 12 h light/dark cycle (lights on: 07.00 h). All experimental procedures used in the present investigation were carried out in accordance with the National Institute of Health 'Guide for the Care and Use of Laboratory Animals' and approved by our Institutional Protocol Approval Committee in accordance with the Yerkes Primate Research Center Regulations.

Startle apparatus

Four SR-LAB startle response systems (SR-LAB, San Diego Instruments, San Diego, California, USA) were used for training and testing. Each startle device consisted of a clear Plexiglas cylinder (8.8 cm in diameter

and 20.5 cm in length) mounted on a Plexiglas base and placed in a ventilated, sound-attenuated and vibration-attenuated chamber. Each chamber was equipped with a horn Radio Shack speaker (Super Tweeter, Radio Shack, Fort Worth, Texas, USA) mounted 24 cm above each cylinder, which was used to present a background white-noise stimulus and 50-ms noise-burst startle stimuli at intensities ranging from 90 to 120 dB. A 15-W light bulb attached 24 cm above each cylinder was used to provide the light stimulus. The footshock unconditioned stimulus (US) was delivered through a removable stainless steel grid floor using one of four LeHigh Valley shock generators (SGS-004; LeHigh Valley, Beltsville, Maryland, USA) located outside the sound-attenuating chamber. Movements within the cylinder created changes in voltage as detected by a piezoelectric accelerometer attached to the Plexiglas base. Voltage output signals were rectified, amplified, and digitized on a 0–4096 unit scale. Startle amplitude was defined as the peak accelerometer voltage that occurred during a 200-ms period beginning at the onset of the startle stimulus. Data acquisition and stimuli deliveries were controlled by a computer using SR-LAB software designed by San Diego Instruments.

To establish a second context used to evaluate the context-specificity of FPS, the SR-LAB startle response system was modified in terms of odor, somatosensory, auditory, and visual cues. The stainless steel grid floors were removed and two chains of 2 cm in length were hung from the top of each cylinder to provide distinctive somatosensory environments. A jar containing a lavender odor was placed inside the sound-attenuated chamber to provide a distinctive olfactory environment. To further differentiate environments, the chamber was supplied with an ambient white-noise stimulus that raised the overall ambient background to 70 dB. In addition, testing and/or training were performed in the chamber illuminated with a light bulb (15 W) located inside the sound-attenuated chamber.

Elevated plus maze

The elevated plus maze consisted of two open arms (50 × 6.5 cm) and two closed arms with a wall (50 × 6.5 × 15 cm) attached to a common central platform (6.5 × 6.5 cm) to form a cross. The maze was elevated 65 cm above the floor. Test sessions were conducted under standard room lighting (100 lx) where behaviors were continuously videotaped by a video camera placed over the apparatus. Before each test, the plus maze was cleaned with Quatricide (Pharmalac, Waterbury, Connecticut, USA).

Handling and animal assignment

Gerbils used in all experiments were accustomed to repeated handling before initiating experimental testing. On each of 3 days before testing, animals were transported

to an experimental room and each animal was handled for approximately 1 min until they appeared to show reduced indicators of stress when handled (e.g. absence of vocalization and struggling). After this handling period, each animal was weighed and the tail was inked with permanent marker to designate subject number before returning to colony housing.

Gerbils used to determine the floor and ceiling effects to various startle intensities were also used to examine the effects of GR-205171 on baseline startle responses. Naive animals were used to characterize contextual FPS, evaluate the effects of GR-205171 contextual FPS, and evaluate the effects of GR-205171 on elevated plus maze testing.

Evaluation of the effects of GR-205171 on baseline startle responses

For 2 consecutive days, each gerbil was acclimated to the startle cylinder for 20 min during which time no startle stimuli were presented and then they were returned to their home cage. The next day, gerbils were placed in the cylinder and after 5 min given 10 startle stimuli at each of four different startle stimulus intensities (90, 100, 110, and 120 dB) with an interstimulus interval of 30 s. All startle stimuli were presented in a pseudorandom order with the constraint that each stimulus intensity occurred only once in each consecutive four-trial block. Ten blocks were presented for a total of 40 trials. This 40-trial startle-stimulus session was used to determine the floor and ceiling effects to various startle intensities, and to enable selection of startle intensities, which produced reliable yet submaximal startle responses for subsequent testing (i.e. 110 and 115 dB).

Gerbils were subsequently matched into four groups with similar mean startle amplitudes to test the effects of GR-205171 on startle responses. The mean startle amplitudes were calculated by averaging the startle amplitude across all earlier 40-test trials. The next day, gerbils were injected intraperitoneally (i.p.) with GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg) 30 min before receiving a drug startle test. During the drug startle test, animals received a total of 24 startle stimuli at two different intensities (110 and 115 dB) starting after a 5-min acclimation period. The interstimulus interval was 30 s and the test session was 20 min in duration.

Characterization of contextual fear-potentiated startle in gerbils

The characterization of contextual FPS in gerbils was conducted with a separate cohort of animals using a modified procedure originally designed to validate contextual FPS in rats as described by McNish *et al.* (1997). After handling, 16 animals received a pretraining startle session and were subsequently matched into groups with similar baseline startle amplitudes. During

the pretraining startle session, animals received a total of 24 startle stimuli at two different intensities (110 and 115 dB) starting after a 5-min acclimation period (identical to the drug startle test session described above). The interstimulus interval was 30 s and the test session was 20 min in duration. Half of the animals were tested in chamber A, and the other half in chamber B.

Animals in the 'same group' (A–A and B–B) were trained and tested in the same chamber, whereas animals in the 'different group' (A–B and B–A) were trained and tested in a different chamber. The assignment of animals to groups and chambers (context A or B) was counter-balanced so that four animals were represented in each of the four possible training and testing conditions. In the training session, gerbils were placed in the startle cylinder and after 5 min received 10 unsignaled footshocks (0.6 mA) at a 2-min variable interstimulus interval (range, 1–3 min). The training session was 25 min in duration, after which gerbils were returned to their home cages. Twenty-four hours after training, animals received a posttraining test that was identical to the pretraining startle session.

Evaluation of the effects of GR-205171 contextual fear-potentiated startle in gerbils

After handling, naive gerbils were first given a pretraining startle session in context A and matched into groups with similar startle amplitudes. One day later, gerbils were given unsignaled footshock training sessions on each of 2 consecutive days. Two days of training sessions were given to increase the FPS levels. The next day, gerbils were injected i.p. with GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg) 30 min before they were tested for startle in context A. This test was identical to the pretraining startle session.

Evaluation of the effects of GR-205171 on elevated plus maze testing

After handling, naive gerbils were injected i.p. with GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg) 30 min before elevated plus maze testing. At the start of each session, one gerbil was placed at the distal end of a closed arm with their heads facing the wall – rather than the central portion of the maze – to prevent possible ambiguous or biased arm entries. Animals were allowed to explore freely for 5 min. The percentage of open arm entries [open arm/(open + closed arm) entries] \times 100 and percentage time in open arms [time in open arms/(time in open + closed arms)] \times 100 were computed. Both of these parameters are indicators of anxiolytic-like activity (Pellow and File, 1986; Hogg, 1996). The total number of closed arm entries was used as an indicator of locomotor activity (Rodgers and Dalvi, 1997). Arm entry was considered complete if all four paws entered a closed or open arm from the central platform. In addition to these standard parameters, we also measured the number of entries and

the amount of time spent by the animals in the center platform. Center time and entries accumulated when at least one paw was placed out of an arm.

Drug preparation

The NK1 antagonist GR-205171 (GlaxoSmithKline Pharmaceuticals, King of Prussia, Pennsylvania, USA) was administered i.p. in PBS at a volume of 5 ml/kg 30 min before testing. Past research has shown GR-205171 to have high affinity and selectivity for NK1 receptors (Gardner *et al.*, 1996). Doses and pretreatment times were based on data from autoradiography and behavioral experiments showing that the doses selected for this study, 0.3, 1.0, and 5.0 mg/kg, are sufficient to linearly inhibit 125 I-SP binding approximately 25–75% in striatal homogenates of gerbils (IC_{50} = 1.25 mg/kg) and significantly inhibit NK1 agonist-induced foot drumming in gerbils (Duffy *et al.*, 2002).

Data reduction and analyses

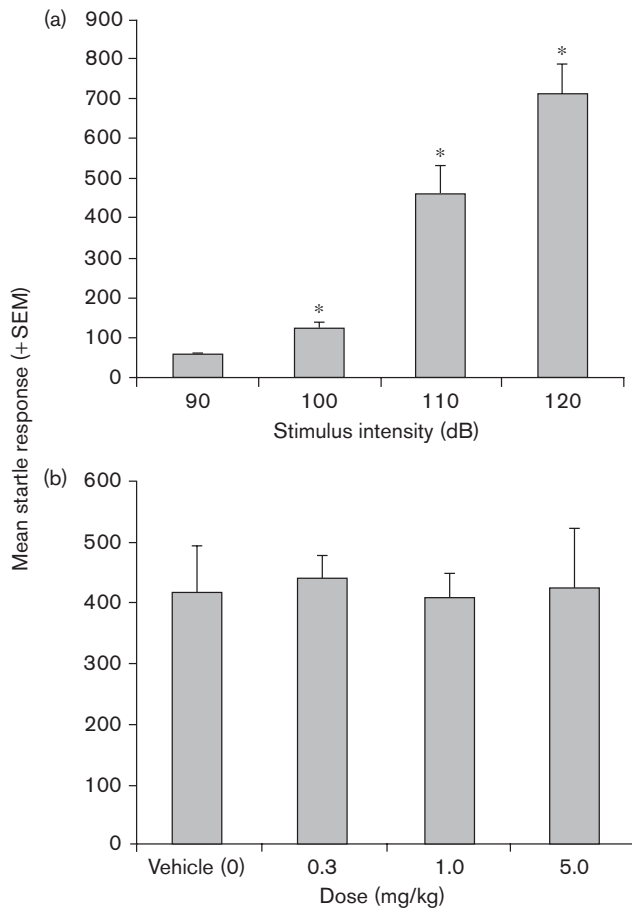
Mean startle response elicited at increasing noise-burst intensities were calculated by averaging the startle amplitude at each intensity (90, 100, 110, and 120 dB). Mean startle amplitudes for the pretraining and posttraining test sessions were calculated by averaging the startle amplitude across all test trials. In cases where analyses were conducted on the first and/or second halves of the posttraining test, mean startle responses were calculated by averaging the startle amplitude across trials 1–12 or trials 13–24, respectively. Block values represented in figures denote the mean startle response of two sequential test trials of different intensity (110 and 115 dB). FPS was detected by comparing pretraining mean startle amplitudes to posttraining mean startle amplitudes. Significant FPS was defined as a reliable increase from pretraining to posttraining startle. Therefore, group differences were examined using repeated-measures analyses of variance (ANOVAs), with session (pretraining, posttraining) as the within-subject variable, or paired *t*-tests. Group differences were also evaluated with simple effects at each level of session. Each elevated plus maze-dependent measure was examined using a one-way ANOVA. Significant mean group differences were detected by use of using Bonferroni's *t*-tests to control the experimentwise error rate at α = 0.05.

Results

Effects of GR-205171 on baseline startle response

Figure 1a shows the mean startle responses elicited at increasing noise-burst intensities in gerbils. Startle amplitudes increased as a function of stimulus intensity, as confirmed by a repeated-measures ANOVA that showed a significant intensity effect [$F(4,95)$ = 20.78; P < 0.001]. Bonferroni's *t*-tests showed significant differences among all intensities (P < 0.001), and a trend analysis indicated that the linear component accounted for a largest and significant proportion of the variance

Fig. 1



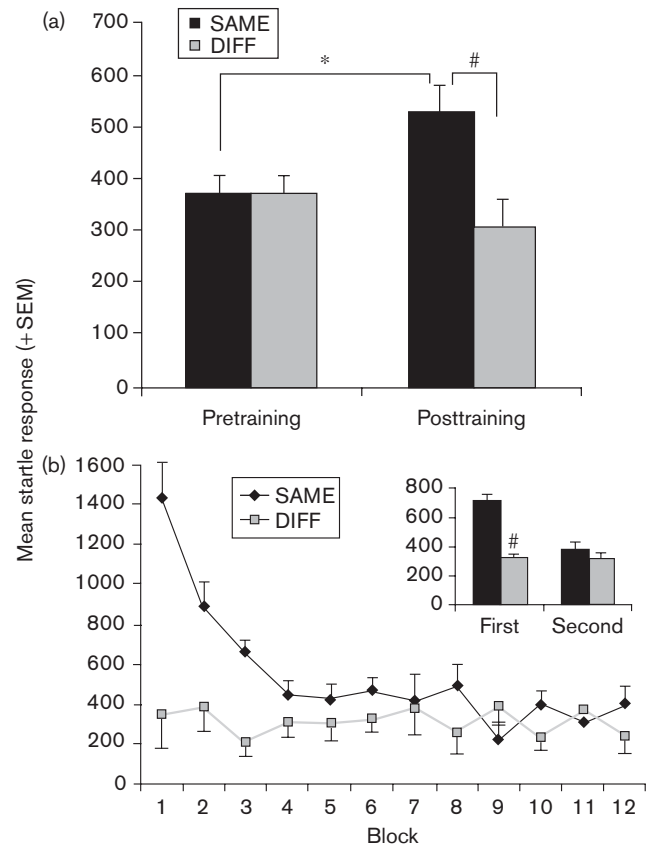
(a) Mean startle responses elicited at increasing noise-burst intensities in gerbils ($N=20$). Startle responses in gerbils increased as a function of stimulus intensity (90, 100, 110, 120 dB). (b) Effects of the neurokinin (NK)1 antagonist GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg, intraperitoneally) on mean startle responses in gerbils. Gerbils received treatment of GR-205171 at 0, 0.3, 1.0, or 5.0 mg/kg doses ($n=5$ /group) 30 min before testing in which animals received startle stimuli at two different intensities (110 and 115 dB). Error bars denote 1 standard error of the mean (SEM). Bonferroni's t -tests showed significant startle differences among all intensities. * $P<0.001$.

($\eta^2 = 0.73$; $P < 0.001$). As shown in Fig. 1b, none of the doses of GR-205171 altered mean baseline startle responses when gerbils were subsequently given a test in which startle was elicited with two different noise-burst intensities (110 and 115 dB). The lack of drug effect on startle was verified by a one-way ANOVA which yielded no significant main effect of drug dose [$F(3,19) = 0.04$; NS].

Contextual fear-potentiated startle in gerbils

Figure 2a shows the mean pretraining and posttraining startle response of gerbils in the same-context (A–A and B–B) and different-context (A–B and B–A) groups. Animals trained and tested in the same context displayed greater mean startle responses after training relative to

Fig. 2



(a) Mean pretraining and posttraining startle response of gerbils trained and tested in the same (SAME) context ($n=8$; A–A and B–B) or different (DIFF) contexts ($n=8$; A–B and B–A). (b) Mean startle responses of gerbils across blocks during the posttraining startle test. Block values represent the mean startle response of two sequential test trials of different intensity (110 and 115 dB). The inset panel displays the mean startle responses during the 'First' and 'Second' halves of the posttraining test. *Significant fear-potentiated startle as defined as a reliable increase from pretraining to posttraining startle ($P < 0.05$). #Significant group differences ($P < 0.02$). The error bars denote 1 standard error of the mean (SEM).

before training, indicating significant FPS to the context; whereas, gerbils trained and tested in different contexts showed no FPS. Figure 2b shows the mean startle responses of gerbils across blocks during the posttraining test. The significant difference in startle responses between groups gradually declined across the test session, and comparatively little differences in startle amplitudes could be detected during the second half of the test session. Altogether, these findings suggest that gerbils tested in the same context, but not in a different context, displayed significant contextual FPS, and within-session extinction of contextual FPS.

An overall ANOVA using group (same, different) as a between-subjects factor and session (pretraining, posttraining) as a within-subjects factor showed a significant main effect of group [$F(1,14) = 4.73$; $P < 0.05$] and a

significant Group by Session interaction [$F(1,14) = 10.96$; $P < 0.01$]. Subsequent paired t -tests with Bonferroni's corrections indicated that the same-context group displayed greater mean startle amplitudes after training relative to before training [$t(7) = 2.99$; $P < 0.02$], whereas the different-context group showed no differences in startle between sessions [$t(7) = 1.69$; NS]. Same-context and different-context groups displayed similar startle amplitude during the pretraining startle sessions [$t(14) = 0.92$; NS].

A closer examination of the posttraining test session showed that during the first half of the test session (blocks 1–6), the gerbils in the same-context group showed greater mean startle responses [$t(14) = 5.07$; $P < 0.05$ (Fig. 2b inset)]. However, during the second half of the test session (blocks 7–12), no reliable differences in mean startle responses were detected [$t(14) = 0.32$; NS]. Thus, although the same-context group showed a greater overall FPS than the different-context group, this effect was mainly mediated by differences during the first half of the posttraining test session.

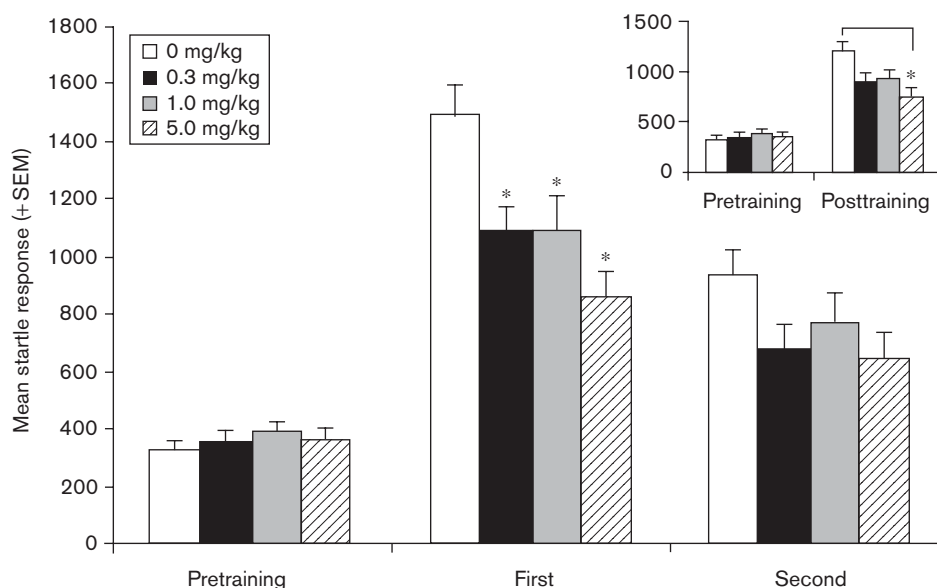
Effects of GR-205171 on contextual fear-potentiated startle

The effects of administration of GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg, i.p.) on contextual FPS are shown in Fig. 3. As greater contextual FPS was observed during the

first half of the posttraining test session, we evaluated the effects of GR-205171 during the first half of the test session, in addition to an analysis of the posttraining session as a whole. Figure 3 shows the mean startle response of groups before training, and their mean startle responses during the first and second halves of the posttraining test (the inset displays posttraining session as a whole). Overall, all groups displayed greater mean startle responses during the posttraining test session relative to pretraining session, indicating contextual FPS in all groups. However, relative to the vehicle control group (0 mg/kg), groups of animals that received pretreatment of GR-205171 (0.3, 1.0, or 5.0 mg/kg) before the posttraining test displayed less FPS. The reduction of contextual FPS was greatest in the group treated with 5 mg/kg of GR-205171.

An ANOVA using group (0, 0.3, 1.0, or 5.0 mg/kg) and session (pretraining, posttraining) as factors yielded significant main effects of dose [$F(3,23) = 4.05$; $P < 0.05$] and session [$F(1,23) = 151.80$; $P < 0.01$]. More importantly, there was a significant Dose \times Session interaction [$F(3,23) = 5.45$; $P < 0.01$] signifying that test performance was differentially affected by drug dose. Simple one-way ANOVAs at each session level showed a reliable posttraining dose effect [$F(3,23) = 5.46$; $P < 0.01$] but no pretraining dose effect [$F(3,23) = 0.64$; $P < 0.05$]. Follow-up Bonferroni's t -tests showed that FPS in the group

Fig. 3



Effects of the neurokinin (NK)1 antagonist GR-205171 on contextual fear-potentiated startle. Gerbils were administered GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg, intraperitoneally) 30 min before the posttraining test session. The bar graph presents the mean startle response of groups before training and their mean startle responses during the 'First' and 'Second' halves of the posttraining test. The inset panel displays the startle responses of gerbils across blocks during the posttraining startle test. Block values represent the mean startle response of two sequential test trails of different intensity (110 and 115 dB). For each group, the mean startle response (collapsed across the posttraining test session) with standard error of the mean (SEM) in parentheses was the following: 0 mg/kg = 1216 (73), 0.3 mg/kg = 882 (78), 1.0 mg/kg = 931 (103), and 5.0 mg/kg = 753 (86).

*Significant difference from vehicle (0 mg/kg) group ($P < 0.05$). Error bars denote 1 SEM.

treated with 5 mg/kg of GR-205171 was significantly less ($P < 0.01$) than in the vehicle control group (0 mg/kg; Fig. 3 inset). No other t -test was statistically significant.

The planned analysis of FPS levels during the first half of the test session (blocks 1–6) also yielded a reliable posttraining dose effect [$F(3,23) = 8.06$; $P < 0.01$]. Follow-up Bonferroni's t -tests indicated that all groups that received GR-205171 (0.3, 1.0, and 5.0 mg/kg) displayed less FPS than the vehicle control group ($P < 0.05$; Fig. 3). Thus, although the highest dose of GR-205171 (5 mg/kg) reduced overall FPS (blocks 1–12), lower doses (0.3 and 1.0 mg/kg) also induced a reduction of FPS, which could be detected during the first half of the posttraining test session when FPS expression was greatest (blocks 1–6).

The effects of GR-205171 on elevated plus maze testing

An evaluation of the percentage time in open arms showed a significant treatment effect [$F(3,30) = 5.14$; $P < 0.01$]. As shown in Fig. 4a, there was a significant relationship between drug dose and percentage time in open arms [$F(1,30) = 14.78$; $P < 0.01$]. Individual contrasts showed that treatment with 5 mg/kg of GR-205171 increased percentage time in open arms [$t(19) = 3.86$; $P < 0.01$]. The comparison between vehicle (0 mg/kg) and 1.0 mg/kg groups was marginally nonsignificant [$t(19) = 1.79$; $P = 0.08$], and the difference between vehicle and 0.3 mg/kg groups was nonsignificant [$t(19) = 1.01$; NS].

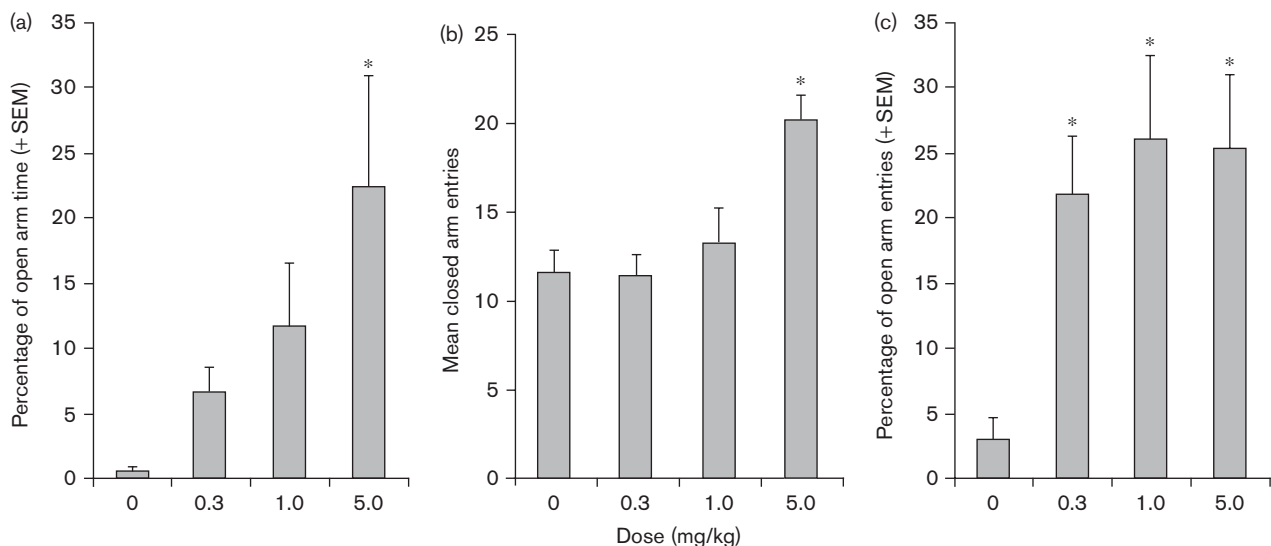
Assessment of total closed arm entries indicated significant differences in locomotor activity among

treatments groups [$F(3,30) = 8.33$; $P < 0.01$]. Individual contrasts comparing the vehicle group with each GR-205171 treatment group indicated that gerbils treated with the high dose of GR-205171 (5.0 mg/kg) made significantly more closed arm entries than the vehicle group, indicating a drug-induced increase in exploratory/motor-stimulant activity [$t(19) = 4.39$; $P < 0.01$]. No significant differences in number of closed arm entries were observed between the vehicle, 0.3 and 1.0-mg/kg treatment groups (all NS, Fig. 4b). To factor out this increase in locomotor activity, the data were analyzed using percentage open arm entries. At doses of 0.3, 1.0, and 5.0 mg/kg, GR-205171 caused a significant anxiolytic-like effect as shown by significant increases in the percentage open arm entries. As shown in Fig. 4c, all three treatment levels of GR-205171 reliably increased the percentage open arm entries as shown by a significant one-way ANOVA [$F(3,30) = 8.45$; $P < 0.01$], and individual contrasts comparing the vehicle group with subsequent treatments groups were all significant ($P < 0.01$). No other t -test was statistically significant. Group means for the number of open, total, and center entries, and center arm time are presented in Table 1.

Table 1 Effects of GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg) on the behavioral measures from the elevated plus maze

Dose (mg/kg)	No. of open entries (SEM)	No. of total entries (SEM)	No. of center entries (SEM)	Center time (s)
0.0	0.9 (0.2)	12.8 (1.3)	14.1 (1.1)	102.5 (6.1)
0.3	3.1 (0.3)	14.6 (1.0)	12.7 (1.1)	117.4 (9.7)
1.0	5.5 (1.5)	18.8 (2.8)	11.3 (1.1)	111.7 (9.8)
5.0	8.3 (2.5)	28.1 (3.0)	12.8 (1.9)	82.4 (9.9)

Fig. 4



Effects of the neurokinin (NK)1 antagonist GR-205171 on elevated plus-maze performance. Gerbils were administered GR-205171 (0, 0.3, 1.0, or 5.0 mg/kg, intraperitoneally) 30 min before testing. (a) Percentage of open arm time. (b) Number of closed arm entries. (c) Percentage of open arm entries on the elevated plus-maze. *Significant difference from vehicle (0 mg/kg) group ($P < 0.05$). Error bars denote 1 standard error of the mean (SEM).

Discussion

This study sought to determine whether systemic administration of the NK1 receptor antagonist GR-205171 in gerbils produced anxiolytic-like effects in the elevated plus maze and a novel contextual conditioned fear test using the FPS paradigm. In the contextual FPS test, gerbils displayed reliable increases in startle when tested in a context previously associated with unsignaled footshocks. In contrast, earlier unsignaled footshock training did not augment startle if gerbils were tested in a different context, indicating context-specific conditioning. When gerbils were tested for FPS after GR-205171 administration, all drug doses (0.3, 1.0, and 5.0 mg/kg) significantly attenuated contextual FPS when compared with the vehicle control group.

Data from earlier studies conducted with laboratory mice and rat show that reference anxiolytics, such as benzodiazepine agonists, effectively reduce the expression of both cue-specific (Guscott *et al.*, 2000; Risbrough *et al.*, 2003) and contextual FPS (Young *et al.*, 1991; Joordens *et al.*, 1997; Guscott *et al.*, 2000), thus supporting the use of the FPS paradigm to investigate anxiolytic properties of drugs. Independent of the anxiolytic-like effects, benzodiazepine agonists also attenuate baseline startle amplitudes in a dose-dependent manner (Joordens *et al.*, 1998). In contrast, none of the doses of GR-205171 tested altered baseline startle amplitude in this study. Future studies will need to explore the behavioral profile of anxiolytics, such as benzodiazepines, serotonin agonists, and other NK1 antagonists in gerbils using the FPS paradigm.

In the elevated plus maze, GR-205171 produced an anxiolytic-like effect as indexed by an increase in the percentage time spent in the open arms and percentage of open arms entries. In the case of percentage open arms entries, all three doses of GR-205171 produced significant increases as compared with the control vehicle group. GR-205171 administration increased percentage open arm time in a dose-dependent manner, causing a significant increase above the control group at the highest dose (5.0 mg/kg). The highest dose of GR-205171, however, also increased locomotor activity as reflected by the total number of closed arms (Cruz *et al.*, 1994; Rodgers and Johnson, 1995; Rodgers *et al.*, 1997). It is often assumed that increases in open arm indices reflect a specific effect on fear/anxiety, provided there is no simultaneous change in locomotor activity. The increase in closed arm entries could reflect a nonspecific arousal/locomotor stimulation resulting in potential 'false positives'. However, the increase in open arm entries observed at lower dose levels (0.3 and 1.0 mg/kg) were not accompanied by significant locomotor effects. Furthermore, no group differences were detected in center hub behaviors, which have been used as indices of

exploratory motivation (Lee and Rodgers, 1991; Rodgers *et al.*, 1992). Thus, whether changes in open arm time reflect an anxiolytic-like effect or some other processes independent of the fear, such as general arousal or an enhanced motivation to explore, is unclear. However, reference anxiolytics, such as benzodiazepine agonists, which robustly increase open arm indices, also increase locomotion in the elevated plus maze (Lister, 1987; File and Aranko, 1988; Moser, 1989; Rodgers *et al.*, 1992; Derrien *et al.*, 1994; Dawson *et al.*, 1995; Varty *et al.*, 2002b). In contrast, locomotor stimulants, such as amphetamine and caffeine, have inconsistent effects on locomotion in the elevated plus maze (Pellow *et al.*, 1985). Such evidence leads one to question the rationale for excluding compounds that increase closed-area entries.

Varty *et al.* (2002a) have shown earlier that a number of NK1 receptor antagonists produce anxiolytic-like effects in a novel gerbil elevated plus maze. In that study, investigators reported that antagonists produced no enhanced locomotor activity. In addition, open arm indices in vehicle-treated control animals were higher than those in this study. As the plus maze is very sensitive to methodological factors, it is likely that differences in maze construction and test procedures account for the observed discrepancies. For example, testing in our study was carried out on a plus maze elevated higher (65 vs. 35 cm) and with narrower arms (6.5 vs. 8 cm). Furthermore, the maze used by in the study of Varty *et al.* (2002a) had clear closed arms to allow for constant illumination in all parts of the maze and holes incorporated into the Plexiglas floor, to allow gerbils to grip the surface. That study also used female gerbils in contrast to the male gerbils used in this study. In addition to the above factors, animals in our study were tested under higher light conditions (100 vs. 5 lx), which have been shown to reduce open arm entries in gerbils (Varty *et al.*, 2002b). It is possible that any one or combination of these variables could account for the baseline differences observed in our vehicle-treated animals.

In recent years, a number of different behavioral models have been used to assess the anxiolytic-like properties of NK1 receptor antagonists in gerbils. Varty *et al.* (2002b) showed that the elevated plus maze induces an anxiety-like profile and has predictive validity for anxiolytics, such as benzodiazepines, in gerbils. Past research indicates that the NK1 antagonists MK-869; L-742,694; L-733,060; CP-99,994; and CP-122,721 produced anxiolytic-like effects in the gerbil elevated plus maze (Varty *et al.*, 2002a). Consistent with these findings, we have shown that the NK1 antagonist GR-205171 also induces an anxiolytic-like profile in the elevated plus maze without accompanying sedative effects often observed with anxiolytic drugs, such as benzodiazepines (Rupniak *et al.*, 2001).

NK1 antagonists have also been tested against behavioral conditioning paradigms that induce foot drumming, which is observed as a species-specific alarm or fear response in gerbils (Randall, 2001). Ballard *et al.* (2001) have shown that pairing a light-tone conditioned stimulus (CS) with a footshock US produces a robust foot-drumming response during both the conditioning period and after presentation of the CS in a retest 24 h later. Moreover, gerbils treated with the NK1 antagonist MK-869 before conditioning show less shock-induced foot drumming and a significant inhibition of CS-induced foot drumming during subsequent testing. In the same study, Ballard *et al.* (2001) showed that pretreatment with the NK1 antagonist CP-99,994 also produced a significant reduction in foot drumming induced by a 2-mA footshock US. Similarly, exposure to the aversive four-plate fear conditioning apparatus induced foot drumming in gerbils, which could be abolished by either diazepam and the NK1 receptor antagonist L-760735 given before testing (Rupniak *et al.*, 2003b).

Our findings support evidence from other laboratories for anxiolytic activity of NK1 receptor antagonists, and provide a novel Pavlovian conditioned fear test that may be an appropriate procedure to test other NK1 antagonists for preclinical anxiolytic activity in gerbils. The FPS paradigm is widely recognized as a valid measure of fear and anxiety because: (i) humans demonstrate FPS (Grillon and Davis, 1997; Grillon *et al.*, 1999; Ameli *et al.*, 2001), (ii) anxiolytic drugs decrease FPS (Davis, 1979; Patrick *et al.*, 1996; Riba *et al.*, 2001; Grillon *et al.*, 2006; Winslow *et al.*, 2007), and (iii) lesions of brain structures critical for the expression of fear eliminate the expression of FPS (Sananes and Davis, 1992; Campeau and Davis, 1995; Falls and Davis, 1995; Heldt *et al.*, 2000). FPS may be particularly useful for examining conditioned fear in gerbils because fear-induced foot drumming can inhibit the expression of conditioned freezing (Woolley *et al.*, 2006), which many conditioning paradigms use as a behavioral response to index levels of fear and anxiety. It is worth noting, however, that conditioning procedures that induce foot drumming often use higher footshock US intensity than that used in this study. Although not quantified, we did not provoke noticeable foot drumming, presumably because of the relatively low US intensity (i.e. 0.6 mA).

A growing line of evidence indicates that the amygdala is a potential site of action for NK1 receptor antagonists in anxiety. Autoradiographic experiments with NK1 radioligands performed in brain slices of gerbils show a distribution profile that is highly homologous to human, and in both species NK1 receptors are widely distributed in brain regions involved in the regulation of affective behavior and the mediation of stress responses, including various nuclei of the amygdala (Caberlotto *et al.*, 2003;

Rigby *et al.*, 2005; Griffante *et al.*, 2006; Nagano *et al.*, 2006). Earlier studies have also shown that exposure to a variety of aversive stressors such as immobilization and forced swim stress increase SP release in the amygdala, as indexed by microdialysis and corresponding NK1 receptor internalization (Smith *et al.*, 1999; Ebner *et al.*, 2004; Ebner and Singewald, 2005). In contrast, microinjection of NK1 antagonists into the amygdala blocks stress-induced anxiogenic effects (Ebner *et al.*, 2004). Furthermore, the effect of amygdala lesions resembled that observed after the administration of the NK1 antagonists. For example, gerbils that had undergone basolateral and lateral amygdala lesions after aversive four-plate fear conditioning exhibited a release of plate crossings and reduced foot drumming when they were returned to the apparatus 7 days after the initial exposure, resembling effects observed after the administration of the NK1 receptor antagonist, L-760735 (Rupniak *et al.*, 2003b). Bilateral amygdala lesions also blocked footshock-induced immobility (Woolley *et al.*, 2006), and drug-induced foot drumming (Rupniak *et al.*, 2003b).

At present, much less is known about the efficacy of NK1 receptor antagonists as a treatment option for individuals suffering with pathological anxiety; however, several studies have showed that NK1 antagonists, including GR-205171, show promise as anxiolytic and antidepressant drugs in clinical trials (Kramer *et al.*, 2003; Furmark *et al.*, 2005). Recent clinical findings have also shown that SP concentrations are elevated both during and after the symptom-provoking stimulus in the cerebrospinal fluid of posttraumatic stress disorder patients (Geraciotti *et al.*, 2006). Similarly, in a recent PET study using a radioactive analog of GR-205171, Michelgård *et al.* (2007) found that patients viewing phobia-inducing pictures had reduced uptake of the labeled GR-205171 in the amygdala during symptom provocation. This reduction reflected a fear-induced increase in the release of endogenous SP and corresponding reduction in NK1 receptor availability. More recently, Fujimura *et al.* (2009) have shown that compared with healthy subjects, patients with panic disorder have widespread reduction of NK1 receptor binding in brain. Altogether, these findings support the notion that the SP–NK1 receptor system might be an important neurochemical target for the development of selective drugs designed to control pathological anxiety.

Within the context of this study, extensive evidence indicates that the amygdala is critically involved in the acquisition and retrieval of contextual fear conditioning. Amygdala lesions or inactivation block conditioned fear responses to both the context and the explicit cue (Kapp *et al.*, 1984; Davis, 1992; LeDoux, 1994). Studies using freezing as an index of fear have shown that hippocampal lesions made either before training

(Phillips and LeDoux, 1992) or after training (Kim and Fanselow, 1992) also disrupted fear conditioning to the context but not to an explicit cue. However, as assessed by the FPS, the hippocampus may not be as critical as the amygdala for contextual learning (McNish *et al.*, 1997; Gewirtz *et al.*, 2000). Finally, there is published evidence supporting the possibility that augmented startle responses associated with FPS may result from the influence of SP on downstream targets of amygdala. Microinjections of SP into the caudal pontine reticular nucleus (PnC), an important part of the primary acoustic startle circuit, increases the amplitude of the startle response (Krase *et al.*, 1994). This effect can be antagonized by local pretreatment with the NK1 antagonist CP-96,345. Furthermore, sensitization of the startle response by footshocks can also be blocked by local microinjections of CP-96,345 into the PnC (Krase *et al.*, 1994).

In conclusion, this study shows that the NK1 receptor antagonist GR-205171 induced an anxiolytic-like response in the elevated plus maze test. In addition, we have shown that gerbils can display contextual FPS, which can be reduced by pretreatment of GR-205171. These findings support evidence from other laboratories for anxiolytic activity of NK1 antagonists and provide a novel Pavlovian conditioned fear test, which may be an appropriate procedure to test other NK1 antagonists for preclinical anxiolytic activity in gerbils.

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