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Effects of a novel potential antidepressant on the behavior and cortisol levels of isolated guinea pig pups

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Abstract

A novel, potential antidepressant, E-6006 citrate (E-6039), dose-dependently reduced the vocalizations emitted by isolated guinea pig pups. The (+)-E-6006, but not the (−)-E-6006, enantiomer also reduced vocalizing. There were no reliable effects of E-6039 on locomotor activity, crouching, or other behavioral measures, but both E-6039 and the (+)-E-6006 enantiomer elevated plasma cortisol levels during isolation. The contrasting effects of E-6039 on vocalizations and plasma cortisol are discussed in terms of E-6039's putative ability to inhibit release of substance P. The reduction in the vocalizations of isolated guinea pig pups corroborates positive results with this drug in other antidepressant screens utilizing mice and rats, and provides further support for the potential of E-6039 as an antidepressant compound.

Keywords: Antidepressant; Isolation; Vocalizations; Cortisol; Guinea pigs

1. Introduction

E-6006 citrate (5-{a-[2-(dimethylamino)ethoxy]-2-thienylmethyl}-1-methyl-1*H*-pyrazole citrate; i.e., E-6039) is a racemic mixture of (+)-E-6006 citrate (E-6101) and (−)-E-6006 citrate (E-6102) enantiomers being developed as a potential antidepressant. Initial experiments indicate that E-6039 exhibits an antidepressant profile in tests with mice and rats (Fisas et al., 2000). It appears to counter amine depletion as evidenced by its ability to reverse reserpine-induced ptosis in mice. It also was found to reduce "despair" responses of immobility in the tail-suspension test in mice, and during forced swim in rats. E-6039 has been observed to normalize the increased substance P levels in the periaqueductal gray of rats during stressor exposure (Hamon, personal communication), suggesting that the drug may function by inhibiting substance P release.

Involvement of substance P in the regulation of mood and affective disorders appears to be mediated by the NK_1 receptor, which is widely distributed in brain regions underlying emotional behavior (Kramer et al., 1998; Mantyh et al., 1984). However, there appear to be species variants in NK_1 receptors as indicated by an approximately 100-fold lower affinity of the NK_1 receptor for specific antagonists in mice and rats than in humans or other species, including guinea pigs (Beresford et al., 1991; Gitter et al., 1991). Therefore, in evaluating potential antidepressant effects of drugs suspected of acting via substance P systems, it is important to include tests with species other than mice and rats. The guinea pig has proven to be useful for this purpose (Kramer et al., 1998; Watling and Krause, 1993).

The preweaning guinea pig exhibits a strong attraction to its mother (Pettijohn, 1979c; Seward, 1940). Guinea pig pups vocalize at a high rate if briefly isolated in a brightly lit novel cage, but not if they are placed into the cage together with their mother (Hennessy and Ritchey, 1987; Pettijohn, 1979a). The isolation call, or "whistle" (Berryman, 1976), is probably the most extensively studied affective behavioral response in the guinea pig, and has proven to be valuable in examining the neurobiology of "separation distress"

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(Golub, 1995; Harvey et al., 1994; Hennessy et al., 1995; Herman and Panksepp, 1978, 1981; Pettijohn, 1979a). Moreover, the call has been found to be selectively reduced by a variety of antidepressant and anxiolytic medications (Kramer et al., 1998; Molewijk et al., 1996; Pettijohn, 1979b), but not by various other drugs (Kramer et al., 1998; Molewijk et al., 1996), attesting to its utility for screening potential antidepressants. In the most recent of these studies, substance P receptor antagonists under development for their antidepressant potential were also found to reduce the vocalizing of guinea pig pups (Kramer et al., 1998). For these reasons, we chose to examine the ability of E-6039 and its enantiomers to reduce the isolation call of guinea pig pups during brief isolation.

When guinea pig pups are briefly isolated, they also exhibit activation of the hypothalamic–pituitary–adrenal (HPA) system, as indicated by increases in plasma levels of ACTH and cortisol (Hennessy and Moorman, 1989; Hennessy et al., 1989). This response is of interest in the present context because heightened activity of the HPA system is often observed during clinical depression, and has been suggested to be a predisposing factor for the development of some forms of depressive illness (Dinan, 1996; Gold et al., 1988). Findings regarding the effects of substance P on HPA activity have not been consistent across species. Whereas substance P has been observed to elevate HPA activity in humans (Coiro et al., 1992), it appears to reduce HPA activity in rats (Chowdrey et al., 1990, 1995; Jessop et al., 2000; Malendowicz et al., 1996). A secondary objective of the present study was to determine the effect of E-6039 on plasma cortisol concentrations at the conclusion of the isolation procedure.

We also monitored general locomotor activity and the occurrence of a crouched stance. When guinea pig pups are left in isolation for extended periods, an initial, active stage of responsiveness, which is characterized by the vocal response, gives way to a second, passive stage, which is marked by prolonged bouts of a crouched stance. In these bouts, the pup remains virtually motionless for protracted periods, does not vocalize, and holds its body close to the floor while exhibiting extensive piloerection and frequent closure of the eyes (Hennessy et al., 1995). These bouts likely reflect a state of increased, rather than diminished, stress or arousal because pups administered the stress-related neuropeptide CRF 60 min prior to testing will subsequently exhibit all components characteristic of the second stage during the first 30 min of isolation (Hennessy et al., 1995). Thus, reduced vocalizing seen during the second stage of responsiveness to isolation cannot be regarded as a "calming" influence. Previous reports of the effects of antidepressant drugs on the vocalizing of guinea pig pups have not considered the possibility that diminished vocalizing could be due to the drug hastening the onset of the second stage of isolation, as opposed to reducing affective behavior during the first stage. In order to distinguish between these possibilities, we examined whether any reductions in vocalizing achieved by injection of E-6039 were or were not accompanied by prolonged bouts of the crouched stance.

Two experiments were performed. In the first, dose–response relations between E-6039 and behavioral and cortisol responses of guinea pig pups were determined. Experiment 2 examined the relative effect of the two enantiomers of E-6039 (E-6101 and E-6102) on these responses.

2. General methods

2.1. Animals

Adult virgin female guinea pigs (Hartley strain) were purchased at 10 weeks of age and bred in groups of three or four females per male. Visibly pregnant females were rehoused individually in plastic cages ($73 \times 54 \times 24$ cm) with wood chip bedding. Females were checked each morning for births. The day of birth was designated Day 0. The temperature- and humidity-controlled colony room was maintained on a 12/12 h light/dark cycle, with lights on at 0700. Guinea pig chow and water were continuously available. All procedures involving animals were approved by Wright State University's Laboratory Animal Care Committee.

2.2. Drugs, injections, and test procedures

All drugs (E-6039, E-6101, and E-6102) were synthesized at Laboratorios Dr. ESTEVE. Injections (1 ml/kg b.wt.) of drug or saline vehicle were given intraperitoneally 30 min prior to testing. Pups were returned to the home cage for the 30-min pretreatment period. For testing, the pup was carried (< 10 s) in a transport cage from the colony room to the test room. There it was placed into a freshly washed, clear, uncovered, plastic cage ($47 \times 24 \times 20$ cm) under overhead lighting. (Luminance measured at the floor of the cage was 26.2 Cd/m^2.) The behavior of the pup was observed for the next 30 min through one-way glass by a trained observer who was blinded as to experimental condition. Whistle vocalizations were detected via a microphone positioned over the test cage, monitored through headphones, and counted on a hand counter. To assess locomotor activity, the observer recorded the number of times pups crossed lines dividing the floor of the cage into four equal-sized segments. In addition, all test sessions were video-recorded (camera: Panasonic, WV-BP310; video recorder: Panasonic, AG 2550). In Experiment 1, tapes were scored for the number of 60-s intervals in which pups exhibited the crouched stance. The tapes were also examined for any aberrant behaviors (including signs of sedation) or other behavioral outcomes that might discriminate pups of the different conditions. In Experiment 2, these observations were made at the time of testing, and videotapes were then reviewed as needed to confirm observations or to resolve ambiguities.

All testing occurred at Day 14 ± 1day. Generally, no more than one pup from a litter was assigned to a particular condition, though in four cases (three in Experiment 1, one in Experiment 2) two littermates were assigned to the same condition. Any pup used for assessing basal cortisol levels was the first pup to be removed on a particular day. Pups were not returned to the litter after testing. Equal numbers of males and females were included in each experimental group.

2.3. Blood sample collection and cortisol determination

Blood samples were collected by cardiac withdrawal with a standard injection syringe and 23-gauge needle under CO_2 anesthesia. Samples were collected within approximately 2 min. Based on work with other laboratory rodents, this was rapid enough to ensure that the sampling procedure did not elevate cortisol levels in the samples obtained (Coover et al., 1979; Davidson et al., 1968; Riley et al., 1981). Plasma was separated and frozen until assayed for cortisol with a ^{125}I radioimmunoassay kit routinely used in our laboratory (“Coat-a-Count” Diagnostic Products, Los Angeles, CA). Intra-assay coefficients of variation were $< 5\%$.

2.4. Data analysis

For vocalization and plasma cortisol data, multiple comparisons with saline-injected controls were made with the Dunnett’s *t* procedure using a one-way analysis of variance (ANOVA) to derive the estimate of error variance (Dunnett, 1964). For vocalizations, a one-tailed Dunnett’s *t* was used because of the predicted suppressive effect of E-6039 on vocalization rate. In Experiment 1, it was necessary to subject the vocalization data to square root transformation to correct for heterogeneity of variance prior to analysis (raw data are shown in the figure).

For line crossing and crouching, we used nonparametric tests because of a large number of scores of zero in particular conditions. Significant Kruskal–Wallis ANOVA was followed by Mann–Whitney *U* tests to compare results of the saline-injected animals to those of animals in all other conditions. For all measures, patterns across conditions were similar for males and females. For this reason, and because equal numbers of males and females were included in all groups, male and female scores were pooled for analyses.

3. Experiment 1

3.1. Method

There were seven groups of 12 pups each. Pups in the first group were not disturbed until removal from the cage for a blood sample to assess basal plasma cortisol levels. Pups in the other six groups were subjected to the 30-min isolation test followed immediately by collection of a blood sample. The first of these groups was not injected prior to the isolation test. The remaining five groups were injected prior to isolation with either saline vehicle, or 3, 10, 30, or 100 mg/kg b.wt. (expressed as base) of E-6039. All testing was conducted between 1100 and 1530 h. The videotapes of one animal given the 10-mg/kg dose and one given the 30-mg/kg dose could not be scored.

3.2. Results

E-6039 dose-dependently reduced the vocalizing of isolated guinea pig pups (Fig. 1). The two highest doses of E-6039 reduced vocalizing relative to saline by 75% and 95%, respectively. These reductions were significant, $t(66) = 2.36$, 4.09, $P < .05$, .01.

The overall analysis for line crossings was significant, $\chi^2(5) = 11.06$, $P = .05$, reflecting a tendency for less locomotor activity at the higher drug doses, particularly as compared to the noninjected condition. However, paired comparisons showed that the locomotor activity of those pups injected with saline did not differ significantly from that of pups in any other condition (Table 1).

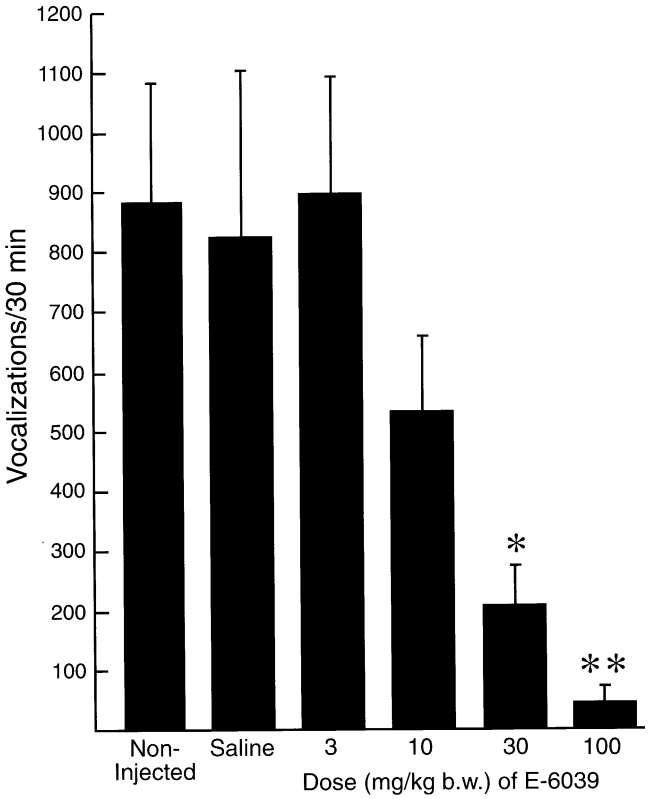

Fig. 1. Mean number of whistle vocalizations by guinea pig pups isolated in a novel test cage for 30 min in Experiment 1. Vertical lines indicate standard errors of the means. * $P < .05$, ** $P < .01$, relative to saline control group (one-tailed tests).

Table 1
Line crossing and crouching by guinea pig pups in the test cage in experiment 1

	Condition					
			Dose (mg/kg b.wt.) of E-6039			
	Noninjected	Saline	3	10	30	100
Line crossing						
% exhibiting	75	58	67	67	25	33
median	10.5	2.0	5.0	3.0	0.0	0.0
Crouching						
% exhibiting	42	42	33	45	45	8
median	0.0	0.0	0.0	0.0	0.0	0.0

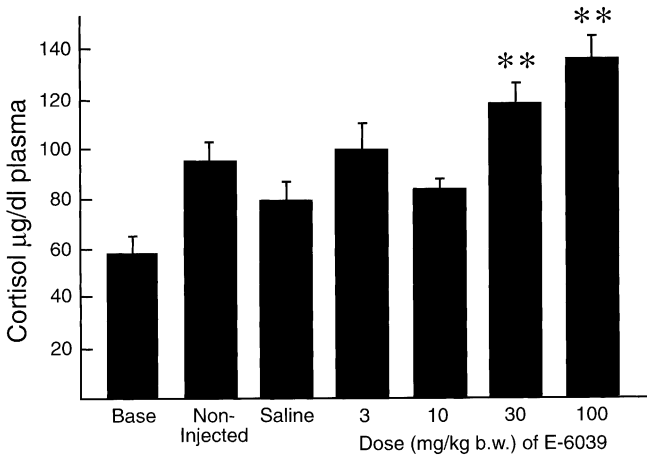

Fig. 2. Mean plasma cortisol levels of pups removed directly from the home cage (base) and of pups isolated in the novel cage for 30 min in Experiment 1. Vertical lines represent standard errors of the means. ** $P<.01$, relative to saline control group.

Fewer than half the pups in any condition ever exhibited a crouched stance. When it did occur, it rarely was protracted (Table 1). Only six pups in the entire experiment exhibited a crouched stance in more than half of the thirty 60-s scoring intervals. The Kruskal–Wallis test confirmed that there was no difference among conditions in crouching. Further inspection of the videotapes revealed no obvious signs of sedation or other aberrant behavior; nor was there any indication that other behavioral outcomes might be associated with administration of E-6039.

As suggested by Fig. 2, there was a stimulatory effect of E-6039 on the plasma cortisol levels of isolated pups. Dunnett's *t* tests showed that the 30- and 100-mg/kg b.wt. doses of E-6039 elevated plasma cortisol levels relative to saline, $t(77)=3.45$, 5.03, Ps $<.01$.

4. Experiment 2

4.1. Method

There were five groups. Pups in the first group ($n=12$) were not disturbed until removal from the cage for a blood sample to assess basal plasma cortisol levels. Pups in the other four groups ($n=16$) were subjected to a 30-min isolation test followed immediately by collection of a blood sample. They were injected prior to isolation with either saline vehicle, or 30 mg/kg b.wt. (expressed as base) of either E-6039 or its enantiomers, E-6101 or E-6102. All testing was conducted between 1130 and 1630 h.

4.2. Results

The 30-mg/kg doses of E-6101 and E-6039 produced 61% and 48% reductions in vocalizations, respectively, relative to the saline control group (Fig. 3). Dunnett's *t* showed that E-6101 significantly reduced vocalizing, $t(60)=2.39$, $P<.05$). The effect of E-6039 was marginally significant in this experiment, $t(60)=1.87$, $P<.09$).

There was no significant difference across conditions for line crossings. Line crossings were exhibited by 62% of saline-injected animals and 44% of animals in each of the drug-injection conditions. Once again, the crouched stance was rarely observed (one to four animals in each condition ever exhibited crouching) and there was no significant difference across conditions for crouching. We observed no obvious signs of sedation and did not identify any other behaviors that might be associated with drug treatment,

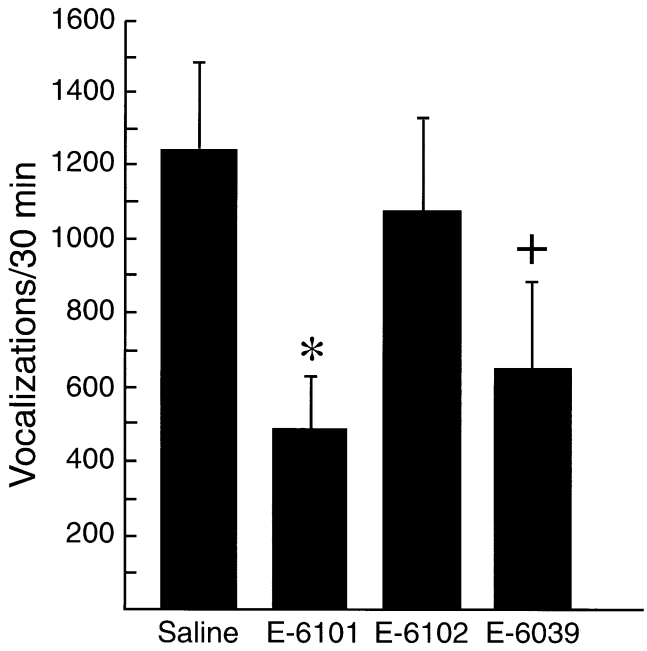

Fig. 3. Mean number of vocalizations by guinea pig pups isolated in a novel test cage for 30 min in Experiment 2. Vertical lines represent standard errors of the means. $^{+}$ $P<.09$, * $P<.05$, relative to saline control group (one-tailed tests).

though three animals (one in each drug condition) exhibited a brief twitching of the eye and head region.

The plasma levels of cortisol of those pups injected with saline and then isolated were significantly elevated over basal levels, $t(71) = 3.91$, $P < .01$. Injection with E-6101 produced a greater elevation than did injection of saline in isolated pups, $t(71) = 2.84$, $P < .05$. The comparison between pups receiving E-6039 and saline was marginally significant, $t(71) = 2.32$, $P < .08$.

5. Discussion

In this study, treatment with E-6039 reduced the vocalizing of isolated guinea pig pups in a dose-dependent fashion. E-6039 did not produce a significant decline in locomotor activity, nor did it lead animals to display any obvious signs of sedation or other unusual behaviors (e.g., persistent freezing) that might have interfered with the vocalization response. The drug did not produce protracted bouts of crouching such as are observed during the second stage of responsiveness during isolation, or following injection with CRF. The little crouching that did occur was not related to drug administration. These results suggest that the lowered vocalization rate achieved by E-6039 constitutes an actual reduction in affective behavior, although competing explanations (e.g., effects of mild sedation) cannot be unequivocally ruled out.

The two-stage response to isolation by guinea pig pups has not been described in other laboratory rodents, but it closely resembles the "protest–despair" stages of separation that have been documented in some primate species (Mineka and Suomi, 1978). As has been suggested for primates (Kaufman and Rosenblum, 1967), the second stage may function largely to conserve resources once immediate reunion with the mother has not been achieved through vocalizing or other active behavior. CRF appears to help trigger the switch between the first and second stages in guinea pig pups. Although injection of ACTH or cortisol does not provoke the onset of the second stage, peripheral injections of CRF and a CRF antagonist hasten and slow, respectively, the onset of the second stage (Hennessy et al., 1991; McInturf and Hennessy, 1996; McConnell and Hennessy, unpublished). Whether the peripherally injected CRF directly accesses the brain to produce its effects (e.g., via the circumventricular organs) remains unresolved (Hennessy et al., 1992). Previous studies of the effects of antidepressant compounds on vocalizing in guinea pig pups have not considered the possibility that vocalizing might be diminished because the drug facilitates the onset of the second stage of isolation, rather than because of a direct influence on affective behavior during the initial stage. Our results indicate that E-6039 does not hasten the onset of the second stage.

The results of Experiment 2 indicate that effects of E-6039 observed here are primarily due to the enantiomer E-6101. For Experiment 2, we chose the lowest dose of E-6039 (30 mg/kg) to produce significant effects in Experiment 1. In Experiment 2, the effects of this dose of E-6039 on vocalizing and plasma cortisol levels fell short of an acceptable level of significance. Nonetheless, the potency of the E-6101 was not dramatically different than that of E-6039. Fig. 4 shows that there was some tendency for E-6102 to elevate cortisol levels, but this increase was not significant.

It is somewhat puzzling that a potential antidepressant would simultaneously reduce affective behavior and raise cortisol levels, particularly in light of the frequently observed elevation in cortisol levels during bouts of clinical depression (Gold et al., 1988). It should be pointed out, however, that the present results are consistent both with findings in guinea pigs that substance P antagonists reduce vocalizing during isolation (Kramer et al., 1998), and with findings in rats indicating that substance P can moderate HPA responses (Chowdrey et al., 1990, 1995; Jessop et al., 2000; Malendowicz et al., 1996). Since substance P has been found to stimulate HPA activity in humans (Coiro et al., 1992), it may be that humans and rodents differ in the direction of the effect of substance P on HPA activity. Furthermore, because the present study examined the effect of a single administration of E-6039, it remains possible that chronic treatment would have a different effect on HPA activity. Several antidepressant agents that work via various modes of action (e.g., monoamine oxidase inhibitor, serotonin reuptake inhibitor) have been found to suppress indices of HPA activity only after chronic administration (Brady, 1994).

We observed a nonsignificant tendency for E-6039 to reduce line crossings. In Experiment 1, only 3 of 12 (25%) and 4 of 12 (33%) pups tested with the two highest doses of E-6039 exhibited any line crossings, whereas 7 of 12 saline-

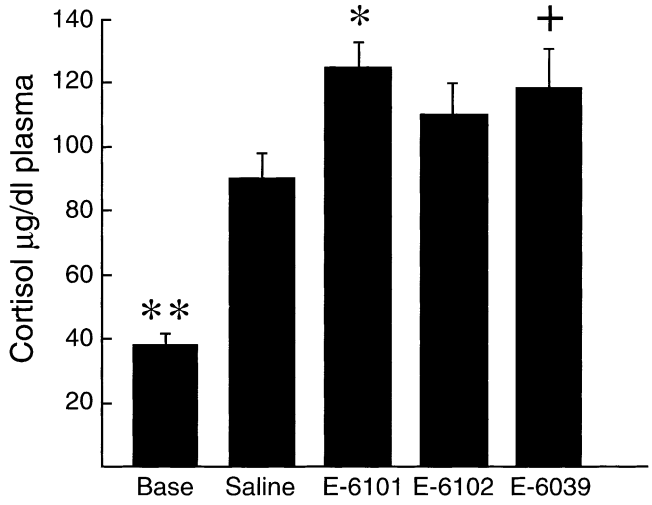

Fig. 4. Mean plasma cortisol levels of pups removed directly from the home cage (base) and of pups isolated in the novel cage for 30 min in Experiment 2. Vertical lines represent standard errors of the means. $^{+}$ $P < .08$, * $P < .05$, ** $P < .01$, relative to saline control group.

injected pups (58%) did so. In Experiment 2, 7 of 16 pups (44%) in each of the three groups exposed to the drug or one of its enantiomers exhibited any line crossings, whereas, 10 of 16 (62%) of saline-injected pups crossed lines. In the guinea pig, substance P has been found to increase locomotor activity (Brent et al., 1988), though cage crossing seems to be less affected than is more complex forms of locomotion (Rupniak and Jackson, 1994). A substance P receptor antagonist was able to reverse increased activity produced by substance P, though administration of the receptor antagonist alone had no moderating influence on locomotor activity and, at high doses, increased activity in a novel cage (McLean et al., 1993). Differences across studies in such factors as route of administration of drugs, details of the behavioral tests, and the age of the test subjects make it difficult to draw firm conclusions regarding the effects of substance P and its antagonists on locomotor activity in guinea pigs.

In summary, we found E-6039, a putative inhibitor of substance P release, to be effective in a behavioral screen for antidepressant medications in the guinea pig. These findings corroborate earlier positive results with the drug in other test paradigms in mice and rats (Fisas et al., 2000), and do so in a species in which substance P systems underlying affective behavior appear to be more similar to those of humans than are those of mice and rats. The results provide further support for the potential of E-6039 as an antidepressant compound. The opposing effects of the drug on the vocalization and cortisol responses raise questions for further study.

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