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Ultrasonic vocalizations of preweanling rats: involvement of both α_2-adrenoceptor and κ-opioid receptor systems

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Abstract

Stimulation of α_2-adrenoceptors and κ-opioid receptors increases the ultrasonic vocalizations of preweanling rats. The purpose of the present study was to determine whether α_2-adrenoceptors and κ-opioid receptors modulate ultrasonic vocalization production via a common mechanism. To that end, 11-day-old rats were injected with the α_2-adrenoceptor antagonist yohimbine (0, 0.5, or 1.0 mg/kg, i.p.) or the κ-opioid receptor antagonist nor-binaltorphimine (0, 5, or 10 mg/kg, i.p.). After 15 min, the same rats were injected with saline, the α_2-adrenoceptor agonist clonidine (0.25 mg/kg, i.p.), or the κ-opioid receptor agonist *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate (U-50,488; 2.5 mg/kg, i.p.). Results showed that both clonidine and U-50,488 increased the ultrasonic vocalizations of preweanling rats. Not surprisingly, clonidine-induced ultrasonic vocalizations were blocked by yohimbine, while U-50,488-induced vocalizations were blocked by nor-binaltorphimine. Importantly, yohimbine also attenuated the vocalizations produced by U-50,488, whereas nor-binaltorphimine did not alter clonidine-induced ultrasonic vocalizations. Thus, it appears that α_2-adrenoceptor and κ-opioid receptor stimulation increases ultrasonic vocalization production via a common mechanism. It is likely that the κ-opioid receptors responsible for modulating ultrasonic vocalizations are located "upstream" from the α_2-adrenoceptors.

Keywords: Ultrasonic vocalization; α_2-Adrenoceptor; κ-Opioid receptor; Clonidine; U-50,488

1. Introduction

Although ultrasonic vocalizations of young rodents have been actively studied since 1956 (Zippelius and Schleidt, 1956), there is a growing debate about the underlying mechanisms responsible for ultrasonic vocalization production (see Hofer and Shair, 1993; Blumberg et al., 2000a). Traditionally, ultrasonic vocalizations have been interpreted as a distress response produced by young animals that are under cold stress or are separated from their dam and littermates (Allin and Banks, 1972; Noirot, 1972; Hofer and Shair, 1978). These ultrasonic vocalizations, in turn, act as signals that elicit maternal behaviors (e.g., pup retrieval) from the dam (Allin and Banks, 1972; Smotherman et al., 1974). More recently, Blumberg and colleagues have challenged this traditional view and have argued that ultrasonic vocalizations are an "acoustic by-product" of a cardiovascular process called the abdominal compression reaction (Blumberg et al., 1999, 2000a,b). According to this interpretation, cold stress causes a reduction in heart rate and venous blood flow. To combat this situation, the young animal involuntarily engages in abdominal compression reactions that result in increased blood pressure and a concomitant increase in ultrasonic vocalizations.

The neural mechanisms mediating ultrasonic vocalizations have been studied intensively, and various neurotransmitter systems have been implicated (for reviews, see Kehoe, 1989; Hård and Engel, 1991). For example, drugs that stimulate α_2-adrenoceptors or κ-opioid receptors dramatically increase ultrasonic vocalization production of young rats (Kehoe and Harris, 1989; Kehoe and Boylan, 1994; Nazarian et al., 1999; Blumberg et al., 2000a);

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whereas, drugs that stimulate μ-opioid or $GABA_A$ receptors decrease ultrasonic vocalizations (Carden and Hofer, 1990; Winslow and Insel, 1991; Fish et al., 2000). In the latter case, available evidence suggests that μ-opioid and $GABA_A$ receptor systems do not interact when modulating ultrasonic vocalizations (Carden and Hofer, 1990). In contrast, it has not yet been determined if α_2-adrenoceptor and κ-opioid receptor systems independently mediate ultrasonic vocalization production, or if they act through a common mechanism. If a common mechanism is involved, it is not known whether the interaction between neurotransmitter systems occurs centrally or peripherally, nor is it known whether stimulating α_2-adrenoceptors and κ-opioid receptors affects ultrasonic vocalization production by modulating distress and anxiety (e.g., Kehoe, 1989; Hård and Engel, 1991), or by altering cardiovascular functioning (e.g., Blumberg et al., 2000a,b).

The goal of this project was to determine whether α_2-adrenoceptor and κ-opioid receptor systems interact when modulating the ultrasonic vocalization production of preweanling rats. To that end, 11-day-old rats were co-administered either yohimbine hydrochloride (an α_2-adrenoceptor antagonist) or nor-binaltorphimine dihydrochloride (a κ-opioid receptor antagonist), along with either clonidine hydrochloride (an α_2-adrenoceptor agonist) or *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate (U-50,488; a κ-opioid receptor agonist). The purpose of using this injection paradigm was to determine whether: (a) the interaction between the α_2-adrenoceptor and κ-opioid receptor systems is "bidirectional" (i.e., both receptor antagonists attenuate the effects of the opposing agonist drug), (b) the interaction is "unidirectional" (i.e., only one receptor antagonist attenuates the effects of the opposing agonist drug), or (c) there is no interaction between the two neurotransmitter systems (i.e., neither receptor antagonist attenuates the effects of the opposing agonist drug). It was originally hypothesized that the interaction between the α_2-adrenoceptor and κ-opioid receptor systems would be "bidirectional" (i.e., yohimbine would attenuate U-50,488-induced ultrasonic vocalizations and nor-binaltorphimine would attenuate clonidine-induced ultrasonic vocalizations). If it is determined that α_2-adrenoceptor and κ-opioid systems interact to modulate ultrasonic vocalization production, then additional studies will be needed to examine whether these drug effects are mediated centrally or peripherally.

2. Materials and methods

2.1. Subjects

Subjects were 175 rat pups of Sprague–Dawley descent (Harlan, Indianapolis, IN, USA), born and raised at California State University, San Bernardino. Litters were culled to 10 rat pups at postnatal day 4 (day 0 = parturition). One rat from each litter was randomly assigned to each treatment group. There was an approximately equal number of male and female rats per group. The colony room was maintained at 22 − 24°C and kept under a 12:12 light/dark cycle. Testing was done in a separate experimental room and was conducted during the light phase of the cycle.

2.2. Apparatus

Ultrasonic vocalizations were assessed in a clear Plexiglas chamber (20 × 20 × 20 cm) housed inside a heated incubator maintained at 34°C (±1°C). A Mini-3 ultrasonic detector (Ultrasound Advice, London, UK) was suspended 8 cm above the floor of the behavioral testing apparatus. The ultrasonic detector was tuned to 40 kHz (±1 kHz), because a setting of 40 − 45 kHz provides the highest rate of ultrasonic vocalization detection (Hofer and Shair, 1978). Ultrasonic vocalizations were recorded and later analyzed by observers blind to drug treatment conditions. Rectal temperatures were assessed using a BAT-12 microprobe thermometer (Physitemp Instruments, Piscataway, NJ, USA).

2.3. Drugs

Clonidine, U-50,488, nor-binaltorphimine, and yohimbine were purchased from Sigma (St. Louis, MO, USA). All drugs were dissolved in saline and injected intraperitoneally (i.p.) at a volume of 5 ml/kg.

2.4. Procedure

2.4.1. Experiment 1

The purpose of the first experiment was to determine a dose of clonidine that would reliably stimulate ultrasonic vocalization production in preweanling rats. To that end, eight litters of 11-day-old rats ($N = 40$) were used. Individual rats from each litter were injected with saline or clonidine (0.05, 0.1, 0.25, or 0.5 mg/kg, i.p.) and returned to the home cage for 15 min. Rats were then taken to a separate experimental room and placed in the testing apparatus. Ultrasonic vocalizations were measured during a 20-min testing session, with rectal temperature being recorded immediately afterwards. As testing on each rat was completed, it was anesthetized using sodium pentobarbital and returned to the home cage. This procedure has the advantage of maintaining litter size (rat pups often begin vocalizing when the number of littermates appreciably declines), while eliminating the ultrasonic vocalizations of the returning rat (see also Carden et al., 1991, 1993). The latter effect is essential, because returning a

vocalizing rat to a litter can stimulate ultrasonic vocalizations in untested rats.

2.4.2. Experiment 2

The goal of Experiment 2 was to determine whether the κ-opioid receptor antagonist nor-binaltorphimine would modify the ultrasonic vocalization production of clonidine- or U-50,488-treated rats. For this purpose, clonidine was administered at a dose of 0.25 mg/kg (based on the results of Experiment 1), whereas U-50,488 was administered at 2.5 mg/kg. This dose of U-50,488 was chosen because a dose range of 1–4 mg/kg produces substantial numbers of ultrasonic vocalizations in preweanling rats (Carden et al., 1994; Kehoe and Boylan, 1994; Nazarian et al., 1999). Eight litters of 11-day-old rats ($N = 72$) were used. Individual rats from each litter were injected with nor-binaltorphimine (0, 5, or 10 mg/kg, i.p.) and placed back in the home cage. After 15 min, rats were injected with saline, U-50,488 (2.5 mg/kg, i.p.), or clonidine (0.25 mg/kg, i.p.) and returned to the home cage for a second time. After an additional 15 min, rats were placed in the testing apparatus and ultrasonic vocalizations were assessed for 20 min (divided into four 5-min time blocks). Rectal temperatures were measured immediately after testing.

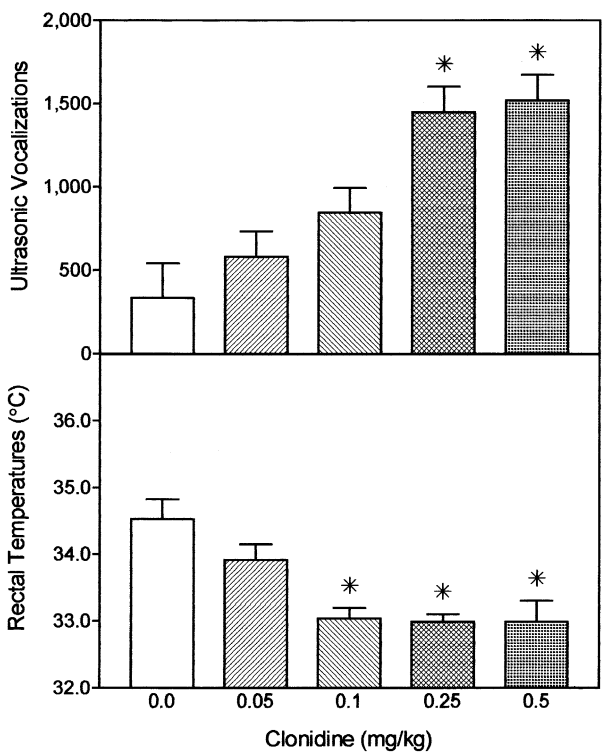

Fig. 1. Mean (±S.E.M.) number of ultrasonic vocalizations (upper graph) and rectal temperatures (lower graph) of 11-day-old rats ($n = 8$ per group) injected with clonidine (0.0, 0.05, 0.1, 0.25, or 0.5 mg/kg, i.p.), 15 min prior to being placed in the testing apparatus. Ultrasonic vocalizations were assessed for 20 min, with rectal temperatures being measured immediately afterwards. * Significantly different from rats given 0.0 mg/kg clonidine, $P < 0.05$.

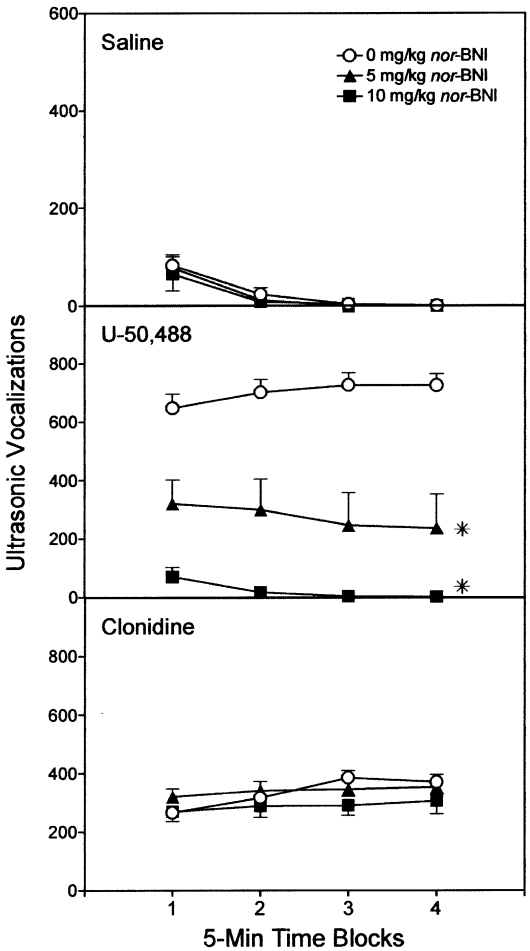

Fig. 2. Mean (±S.E.M.) number of ultrasonic vocalizations of 11-day-old rats ($n = 8$ per group) injected with nor-binaltorphimine (nor-BNI; 0, 5, or 10 mg/kg, i.p.) 30 min prior to behavioral testing, and saline (upper graph), 2.5 mg/kg U-50,488 (middle graph), or 0.25 mg/kg clonidine (lower graph) 15 min prior to testing. Ultrasonic vocalizations were assessed for 20 min (divided into four 5-min time blocks). * Significantly different from similarly treated rats given 0 mg/kg nor-BNI (open circles), $P < 0.05$.

2.4.3. Experiment 3

Results of Experiment 2 showed that clonidine-induced ultrasonic vocalizations were not modified by nor-binaltorphimine. To extend these findings, an additional experiment was conducted in which we determined whether the α_2-adrenoceptor antagonist yohimbine would modify the ultrasonic vocalization production of U-50,488- or clonidine-treated rats. Seven litters of 11-day-old rats ($N = 63$) were used. Individual rats from each litter were injected with yohimbine (0, 0.5, or 1.0 mg/kg, i.p.) and placed back in the home cage. After 15 min, rats were injected with saline, U-50,488 (2.5 mg/kg, i.p.), or clonidine (0.25 mg/kg, i.p.) and returned to the home cage for a second time. After an additional 15 min, rats were placed in the testing apparatus and ultrasonic vocalizations were assessed for 20 min (divided into four 5-min time blocks).

Rectal temperatures were measured immediately after testing.

2.5. Statistics

Analyses of variance (ANOVAs) were used for statistical analysis of ultrasonic vocalization and rectal temperature data. For each of these analyses, litter effects were controlled by using within-litter statistical procedures (i.e., a within analysis using one value/condition/litter) (Zorrilla, 1997). In Experiment 1, ultrasonic vocalization and rectal temperature data were analyzed using one-way (treatment) ANOVAs. In Experiments 2 and 3, ultrasonic vocalization data were analyzed using $3 \times 3 \times 4$ (pretreatment $\times$ treatment $\times$ time) ANOVAs; whereas, rectal temperature data were analyzed using 3×3 (pretreatment $\times$ treatment) ANOVAs. Post hoc analysis of the data was made using Tukey tests ($P < 0.05$). Separate between-subject ANOVAs indicated that mean number of ultrasonic vocalizations did not differ according to gender, so data were presented collapsed across the sex variable. The lack of statistically reliable gender differences was not surprising, because it is commonly reported that male and female preweanling rats exhibit similar levels of basal and drug-induced ultrasonic vocalizations (Meyer and Yacht, 1993; Dastur et al., 1999; Nazarian et al., 1999).

3. Results

3.1. Effects of clonidine on ultrasonic vocalizations

Clonidine produced a dose-dependent increase in the ultrasonic vocalization production of 11-day-old rats (upper graph, Fig. 1); treatment effect, $F(4,28) = 14.76$, $P < 0.001$. Specifically, rats given 0.25 or 0.5 mg/kg clonidine emitted more ultrasonic vocalizations than saline controls. Rectal temperatures were also affected by clonidine, as rats injected with higher doses of clonidine (0.1, 0.25, or 0.5 mg/kg) had significantly lower rectal temperatures than saline-treated rats (lower graph, Fig. 1); treatment effect $F(4,28) = 12.24$, $P < 0.001$.

Table 1
Mean (±S.E.M.) rectal temperatures (°C) of 11-day-old rats ($n = 8$ per group) injected with saline, U-50,488 (2.5 mg/kg), or clonidine (0.25 mg/kg) 15 min after being pretreated with nor-binaltorphimine (nor-BNI)

Treatment	Dose of nor-BNI (mg/kg)		
	0	5	10
Saline	34.75 (±0.41)	35.29 (±0.35)	35.18 (±0.39)
U-50,488	34.72 (±0.18)	34.66 (±0.35)	34.54 (±0.35)
Clonidine	33.34 (±0.19)[a]	33.19 (±0.24)[a]	33.34 (±0.29)[a]

[a] Significantly different from saline-treated rats ($P < 0.05$).

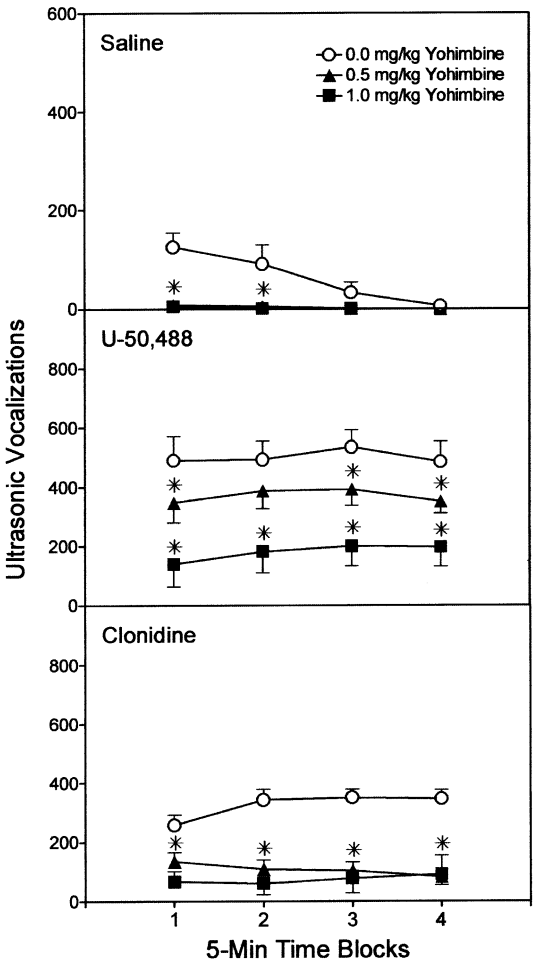

Fig. 3. Mean (±S.E.M.) number of ultrasonic vocalizations of 11-day-old rats ($n = 7$ per group) injected with yohimbine (0, 0.5, or 1.0 mg/kg, i.p.) 30 min prior to behavioral testing, and saline (upper graph), 2.5 mg/kg U-50,488 (middle graph), or 0.25 mg/kg clonidine (lower graph) 15 min prior to testing. Ultrasonic vocalizations were assessed for 20 min (divided into four 5-min time blocks). * Significantly different from similarly treated rats given 0 mg/kg yohimbine (open circles), $P < 0.05$.

3.2. Effects of nor-binaltorphimine on clonidine- and U-50,488-induced ultrasonic vocalizations

Overall, U-50,488- and clonidine-treated rats emitted more ultrasonic vocalizations than saline controls (Fig. 2); treatment main effect, $F(2,14) = 55.91$, $P < 0.001$. κ-Opioid receptor blockade differentially affected ultrasonic vocalization production depending on the type of receptor agonist given (i.e., an α_2-adrenoceptor or κ-opioid receptor agonist). More specifically, nor-binaltorphimine caused a dose-dependent reduction in U-50,488-induced ultrasonic vocalizations (middle graph, Fig. 2), while leaving clonidine-induced ultrasonic vocalizations unaffected (lower graph, Fig. 2); pretreatment $\times$ treatment interaction, $F(4,28) = 20.87$, $P < 0.001$. nor-Binaltorphimine did not affect the ultrasonic vocalizations of saline-treated rats (upper graph, Fig. 2).

Table 2
Mean (±S.E.M.) rectal temperatures (°C) of 11-day-old rats ($n = 7$ per group) injected with saline, U-50,488 (2.5 mg/kg), or clonidine (0.25 mg/kg) 15 min after being pretreated with yohimbine

Treatment	Dose of yohimbine (mg/kg)		
	0.0	0.5	1.0
Saline	34.97 (±0.35)	34.40 (±0.41)	34.84 (±0.32)
U-50,488	34.51 (±0.36)	34.71 (±0.18)	34.44 (±0.40)
Clonidine	33.23 (±0.31)[a]	33.69 (±0.31)[a]	33.89 (±0.46)[a]

[a] Significantly different from saline-treated rats ($P < 0.05$).

As in Experiment 1, clonidine (0.25 mg/kg) decreased the rectal temperatures of 11-day-old rats, while U-50,488 had no effect (Table 1); treatment main effect, $F(2,14) = 30.21$, $P < 0.001$. nor-Binaltorphimine did not modify the clonidine-induced reduction in rectal temperatures.

3.3. Effects of yohimbine on clonidine- and U-50,488-induced ultrasonic vocalizations

Once again, rats given U-50,488 or clonidine emitted more ultrasonic vocalizations than saline controls (Fig. 3); treatment main effect, $F(2,12) = 49.71$, $P < 0.001$. Importantly, the α_2-adrenoceptor antagonist yohimbine reduced the ultrasonic vocalization production of saline-, U-50,488-, and clonidine-treated rats; pretreatment × treatment × time interaction, $F(12,72) = 2.94$, $P < 0.01$. More specifically, yohimbine (0.5 or 1.0 mg/kg) decreased the ultrasonic vocalizations of saline-treated rats on time blocks 1 and 2 (upper graph, Fig. 3) and clonidine-treated rats on time blocks 1–4 (lower graph, Fig. 3). Yohimbine also caused a dose-dependent reduction in the ultrasonic vocalizations produced by κ-opioid receptor stimulation. The lower dose of yohimbine (0.5 mg/kg) decreased U-50,488-induced ultrasonic vocalizations on time blocks 1, 3, and 4, whereas the higher dose of yohimbine (1.0 mg/kg) decreased U-50,488-induced ultrasonic vocalizations across the entire testing session (middle graph, Fig. 3).

Rectal temperatures of clonidine-treated 11-day-old rats were lower than saline controls (Table 2); treatment main effect, $F(2,12) = 27.00$, $P < 0.001$. Although a trend was apparent, yohimbine did not significantly affect the rectal temperatures of clonidine-treated rats.

4. Discussion

The purpose of the present study was to assess whether the α_2-adrenoceptor and κ-opioid receptor systems stimulate ultrasonic vocalizations through a common mechanism. As reported previously, we found that clonidine (an α_2-adrenoceptor agonist) and U-50,488 (an κ-opioid receptor agonist) increased the ultrasonic vocalization production of preweanling rats (see also Hård et al., 1988; Kehoe and Harris, 1989; Carden et al., 1994; Nazarian et al., 1999; Blumberg et al., 2000a,b). Saline-treated rats emitted a moderate number of isolation-induced ultrasonic vocalizations that decreased as the testing session progressed. As predicted, the α_2-adrenoceptor antagonist yohimbine reduced both clonidine- and U-50,488-induced ultrasonic vocalizations (Fig. 3). In contrast, the κ-opioid receptor antagonist nor-binaltorphimine attenuated U-50,488 (a κ-opioid receptor agonist), but not clonidine-induced ultrasonic vocalizations (Fig. 2). Therefore, it appears that α_2-adrenoceptor and κ-opioid systems interact when mediating ultrasonic vocalization production, but in a more complex manner than originally hypothesized.

Rectal temperatures of rats were also affected by drug treatment. For example, clonidine reduced rectal temperatures of 11-day-old rats in a dose-dependent manner. Ultrasonic vocalizations of clonidine-treated rats generally varied inversely with rectal temperatures (Fig. 1). Clonidine's effects on these two measures were dissociable, however, as yohimbine fully attenuated clonidine-induced ultrasonic vocalizations without significantly altering rectal temperatures (Table 2). The κ-opioid receptor agonist did not affect rectal temperatures, although U-50,488 did increase ultrasonic vocalizations.

When data from this study are considered as a whole, it appears that a functional α_2-adrenoceptor system is necessary for κ-opioid-mediated ultrasonic vocalization production, but the reverse is not the case. Specifically, antagonism of α_2-adrenoceptors attenuated U-50,488-induced ultrasonic vocalizations, while antagonism of κ-opioid receptors did not alter clonidine-induced ultrasonic vocalizations. Thus, the κ-opioid and α_2-adrenoceptor systems interact in a "unidirectional" manner to modulate ultrasonic vocalization production. The most parsimonious explanation is that the κ-opioid receptors responsible for inducing ultrasonic vocalizations are located "upstream" from the critical α_2-adrenoceptors. The exact location of these α_2-adrenoceptors and κ-opioid receptors is unknown, but some pertinent evidence is available.

Although speculative, it is likely that clonidine increases ultrasonic vocalization production in at least one of the following ways. First, clonidine may stimulate α_2-adrenoceptors in brain regions responsible for mediating distress and anxiety (Kehoe and Harris, 1989). Second, α_2-adrenoceptor agonists are capable of decreasing heart rate and blood pressure through central mechanisms (McAuley et al., 1989; Mollace et al., 1989; Aicher and Drake, 1999), so clonidine may indirectly enhance ultrasonic vocalization production by initiating abdominal compression reactions (see Blumberg et al., 2000a,b). Third, stimulation of α_2-adrenoceptors located on postganglionic fibers causes hypotension and bradycardia (Szabo et al., 1989; Wong, 1993), thus, clonidine may induce abdominal compression reactions and a concomitant increase in ultrasonic vocalizations through peripheral mechanisms.

Although κ-opioid receptors are found on postganglionic nor-adrenergic terminals and in heart tissue (Starke et al., 1985; Fuder et al., 1986; Pugsley et al., 1992; Jin et al., 1995), it is likely that U-50,488 enhances ultrasonic vocalization production through central actions. Specifically, intracisternal administration of U-50,488 enhances ultrasonic vocalizations of 10-day-old rats (Carden et al., 1991). Whether stimulation of these centrally located κ-opioid receptors affects ultrasonic vocalization production by modulating distress or cardiovascular functioning is uncertain. Work by Barr and colleagues indicates that these κ-opioid receptors may modulate distress, because ultrasonic vocalizations increase after U-50,488 is microinjected into the periaqueductal gray (Goodwin and Barr, 1997): a brain area implicated in both defense responses and vocalizations (Jürgens, 1994; Goodwin and Barr, 1998). Consistent with this finding, the ultrasonic vocalization production of young rats is reduced after bilateral lesions of the periaqueductal gray (Goodwin et al., 1998). Alternatively, stimulation of κ-opioid receptors in the hippocampus, hypothalamus, medulla and, to a lesser extent, the periaqueductal gray decreases heart rate and blood pressure of adult rats (Feuerstein and Faden, 1982; Hassen et al., 1984; Wang and Ingenito, 1994; Keay et al., 1997). Thus, systemic treatment with U-50,488 may stimulate ultrasonic vocalization production by depressing cardiovascular functioning. When considered together, it remains uncertain whether U-50,488 increases ultrasonic vocalization production by affecting distress, cardiovascular processes, or both.

In conclusion, administration of an α_2-adrenoceptor antagonist attenuates the ultrasonic vocalization production caused by κ-opioid receptor agonist treatment. This suggests that the κ-opioid receptors responsible for modulating ultrasonic vocalizations are "upstream" from the α_2-adrenoceptors. One possibility is that α_2-adrenoceptor stimulation increases ultrasonic vocalizations by depressing cardiovascular functioning and initiating abdominal compression reactions (see Blumberg et al., 2000a,b). It is likely that κ-opioid receptor agonists increase ultrasonic vocalization production via central mechanisms (see Carden et al., 1991). Whether these κ-opioid receptors normally modulate distress or cardiovascular functioning is unclear. What seems certain, however, is that κ-opioid receptor stimulation is only able to induce ultrasonic vocalizations if the α_2-adrenoceptor system is functional. The opposite cannot be said, because clonidine-induced ultrasonic vocalizations were unaffected by κ-opioid receptor blockade.

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