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Ultrasonic vocalizations of preweanling rats the interaction of k-opioid and a_2-noradrenergic systems

Arbi Nazarian

ULTRASONIC VOCALIZATIONS OF PREWEANLING RATS: THE INTERACTION OF κ-OPIOID AND α_2-NORADRENERGIC SYSTEMS

A Thesis

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ABSTRACT

The purpose of this thesis was to determine whether κ-opioid and α_2-noradrenergic systems interact when mediating the ultrasonic vocalizations (USV) and locomotor activity of preweanling rats. In order to determine whether these neurotransmitter systems interact, rats were treated with various combinations of κ-opioid and α_2-noradrenergic agonist and antagonist drugs. It was predicted that κ-opioid and α_2-noradrenergic systems would interact to mediate USV production, but not the locomotor activity, of 11-day-old rats. In the first experiment, USVs and line-crosses were measured for 20 min after rat pups were given an injection of saline or the α_2-noradrenergic agonist clonidine (0.05, 0.1, 0.25, or 0.5 mg/kg, ip). Clonidine caused a dose-dependent increase in USV production and reduced locomotor activity. In the second experiment, interaction of the κ-opioid and the α_2-noradrenergic systems was examined by injecting rats with the κ-opioid antagonist *nor*-BNI (0, 5, or 10 mg/kg, ip), followed by an injection of saline, the κ-opioid agonist U50,488 (2.5 mg/kg, ip), or clonidine (0.25 mg/kg, ip). *nor*-BNI reduced U50,488-, but

not clonidine-, induced USV production. *nor*-BNI also decreased U50,488-induced line-crosses. In the third experiment, the interaction of κ-opioid and α_2-noradrenergic systems was further examined by co-administering the α_2-noradrenergic antagonist yohimbine (0.0, 0.5, or 1.0 mg/kg, ip) and saline, U50,488 (2.5 mg/kg), or clonidine (0.25 mg/kg). Interestingly, yohimbine decreased both U50,488- and clonidine-induced USV production. Yohimbine also reduced U50,488-induced line-crosses. These findings confirm that κ-opioid and α_2-noradrenergic systems interact to mediate USV production, however the pattern of the interaction was more complex than originally predicted. More specifically, κ-opioid and α_2-noradrenergic systems interact in a unidirectional manner when mediating USV production, with the κ-opioid receptors located "up-stream" from the noradrenergic neurons.

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Introduction

Animal models of distress and anxiety

Distress and anxiety have been extensively investigated using a variety of animal models. For instance, behavioral studies have shown that adult rats often urinate, defecate, and exhibit increased exploratory behaviors, all of which are measures of anxiety in rats (Archer, 1973; Crawley, 1985; Russell, 1973; Walsh & Cummins, 1976). In addition, anxiety reduces the locomotor activity of rats in the open arms of an elevated plus-maze (Pellow, Chopin, File, & Briley, 1985). There are many physiological correlates with anxiety and distress, as anxious rats and mice have enhanced levels of plasma corticosterone and other stress-related hormones (Hennessy & Levine, 1979; Misslin & Cigrang, 1986). Therefore, it appears that anxiety and distress can be assessed in adult rats using both behavioral and physiological measures.

In contrast to adult models, developmental animal models of distress and anxiety are limited due to size of the young rat, physiological immaturity, and inability to perform required tasks. Therefore, more suitable behaviors must be used to accurately assess distress anxiety in young rats. One commonly used measure of distress anxiety is

ultrasonic vocalizations (USV). USVs are often considered to be an emotionally-mediated behavior that may reflect distress in young rodents (Winslow & Insel, 1991a). Nearly 45 years ago, Zippelius and Schleidt (as cited in Hofer & Shair, 1978) found that young mice emit USVs when isolated from the dam. Since then various investigators have examined the behavioral and neurochemical characteristics of ultrasound production in rats and mice.

Characteristics of USVs in young and adult rats.

Since their discovery 45 years ago, USVs have been rigorously studied in neonatal and preweanling rats. USVs produced by young rats range from 30-50 kHz, which is beyond the range of human hearing (Noirot, 1968; Sewell, 1970). Young rats begin emitting USVs shortly after birth, with the frequency of vocalizations increasing until they reach a peak at 10 to 13 days of age. USVs decline soon after this time, and are almost completely absent after 20 days of age (Hofer & Shair, 1978; Kehoe & Harris, 1989; Noirot, 1968). Adult rats also emit USVs, but these calls typically range between 20-24 kHz (Thomas, Takahashi, & Barfield, 1983). These vocalizations are produced as rats engage in copulation and aggressive behaviors (Cagiano et al., 1989; Thomas et al., 1983). Altogether, it appears

that ultrasound production undergoes a number of ontogenetic changes: First, the total number of USVs produced by rats declines across ontogeny and, second, situations resulting in the production of USVs show age-dependent differences.

Distress and cardiovascular models of USVs. In recent years, controversy has developed about the role of USVs in young rodents. Specifically, USVs were originally interpreted to be an emotionally-mediated behavior exhibited by young rats (Noirot, 1968); however, Blumberg and colleagues have challenged this view by providing evidence that USVs are by-products of changes that occur in the cardiovascular system (Blumberg, Sokoloff, & Kent, 1999).

The distress model. In neonatal and preweanling rats, USVs are emitted when the pup is separated from its littermates and dam (Hofer & Shair, 1978; Noirot, 1972). This separation causes the pup to produce ultrasounds that elicit maternal retrieval, caregiving, and aid in thermoregulation (Bell, 1979; Hofer & Shair, 1978; Sales & Smith, 1978; Smotherman et al., 1974). Thus, separation from the dam and littermates is presumed to be distressing

to the young pup and the resulting vocalizations are interpreted to be a product of their emotional state.

Frequency of isolation-induced USVs can be altered by various factors, such as cold temperature, presentation of the dam, and presentation of an unfamiliar adult male. For instance, rat pups emit USVs when isolated in a cold environment (Allin & Banks, 1971; Blumberg et al., 1998). USVs emitted during cold exposure provoke maternal retrieval for assistance in thermoregulation (Allin & Banks, 1971). Hence, USVs may be emitted to resolve physiological discomfort experienced in a cold environment.

Besides being temperature dependent, USVs can be altered in various social situations. For example, an isolated pup will decrease its vocalizations when an anesthetized dam is presented to the pup (Carden & Hofer, 1992). The decrease in vocalizations is independent of the dam's ability to assist in the thermoregulation of the pup, because pups will decrease their vocalizations even when the dam's body temperature is significantly below normal levels (Carden & Hofer, 1992; Hofer, Brunelli, & Shair, 1993). Upon removal of the dam, the rat pup emits vocalizations at an even greater rate than it did initially (known as potentiation; Hofer, Masmela, Brunelli, & Shair,

1998). However, suppression of USVs and motor movement (i.e., freezing) is observed when an unfamiliar adult male is placed in the same environment with the rat pup (Takahashi, 1992). Interestingly, the isolated rat pup continues to remain quiet after the unfamiliar male is removed (known as inhibition; Shair, Masmela, & Hofer, 1999). The potentiation and inhibition of USVs appears to be emotionally-mediated, because young rats exposed to extreme cold temperatures also increase their USVs or remain quiet upon removal of the dam or an adult male (Shair, Masmela, Brunelli, & Hofer, 1997). In summary, it is evident that isolation-induced USV production can be modulated by various situational factors.

Psychopharmacological manipulations are capable of modulating separation-induced USVs. Drugs that are known to be rewarding (e.g., morphine and cocaine) or anxiety reducing (e.g., diazepam) decrease USVs (Carden & Hofer, 1990; Insel, Hill, & Mayor, 1986; Kehoe & Blass, 1986; Kehoe & Boylan, 1992; Nazarian, Rodarte-Freeman, & McDougall, 1999; Winslow & Insel, 1991b). However, drugs that enhance USVs may do so by having either anxiogenic/aversive properties (Hansen, 1993; Mucha & Herz, 1985) or by directly activating neural pathways that

mediate USV production (Goodwin & Barr, 1997; Jürgens, 1994). For example, the κ-opioid agonist U50,488 and the α_2-noradrenergic agonist clonidine increase the USVs of young rats (Barr, Wang, & Carden, 1994; Blumberg, Sokoloff, Kirby, & Kent, 2000; Hård, Engel, & Lindh, 1988; Kehoe & Boylan, 1994; Kehoe & Harris, 1989; Nazarian et al., 1999). Clonidine- and U50,488-induced vocalizations are not suppressed when the rat pup is placed back in the home cage (Carden, Davachi, & Hofer, 1994; Hansen, 1993), suggesting that these drugs may enhance USVs by stimulating pathways mediating USV production (i.e., these actions may be independent of the drug's aversive or anxiogenic properties).

Various brain regions are involved in the production of USVs. Receptor localization studies have shown the presence of both κ-opioid and noradrenergic receptors in the striatum and amygdala, areas known to be important for affective behaviors. Furthermore, USV production is correlated with Fos-like immunoreactivity in the amygdala, prefrontal cortex, and periaqueductal gray (PAG) of adult rats (Fos is a quick-developing protein that can be used as a regional marker of neuronal activation) (Duncan, Knapp, &

Breese, 1996). Additional evidence suggests that the PAG may be of special importance, as microinjecting U50,488 into the PAG enhances USV production in young rats (Goodwin & Barr, 1997). Finally, the amygdala and PAG are extensively interconnected, suggesting that these two brain regions may jointly mediate some behaviors, perhaps including USVs (Rizvi, Ennis, Behbehani, & Shipley, 1991). Taken together, these results suggest that U50,488 and clonidine may affect USV production by stimulating κ-opioid and noradrenergic receptors in the amygdala and/or PAG.

The cardiovascular model. In the past decade, traditional views about USVs have been challenged by studies questioning the validity of the distress model. More specifically, Blumberg and colleagues argue that USVs are by-products of a cardiovascular process known as the abdominal compression reaction (ACR; Blumberg et al., 1999). The ACR is a mechanism that helps move venous blood back to the heart by the contraction of abdominal muscles during expiration (Youmans, Tjioe, & Tong, 1974). The mechanisms involved in ACR-induced USVs are as follows. An isolated rat pup has difficulty thermoregulating, thus its body temperature decreases when isolated. This decrease

causes heart muscles to cool down and, consequently, both heart rate and blood pressure are reduced (Blumberg, Sokoloff, & Kirby, 1997). The decrease in heart rate causes a reduction of venous flow. Therefore, the ACR assists in propelling the venous blood back to the heart (Blumberg et al., 1999). Ultrasonic vocalizations are, therefore, a by-product of air released by the larynx during expiration.

The young rat has a physiological mechanism that can combat the decrease in body temperature. Rats as well as other mammals have brown adipose tissue (BAT) surrounding critical organs in the body. The role of BAT is to assist in thermoregulation when body temperature decreases (Smith, 1964). Thermoregulation occurs by increasing the metabolism of BAT, resulting in an increase in temperature, which then warms up the critical organs that it surrounds (e.g., the heart; Smith, 1964). The heating of BAT prevents heart muscles from cooling down, thus preventing a decrease in heart rate (Blumberg et al., 1997). The ability of BAT to assist in thermoregulation is limited, because extreme cold temperatures can overwhelm the capacity of BAT to maintain the necessary temperature to prevent the heart from slowing down (Blumberg et al., 1997;

Blumberg & Stolba, 1996). During such situations, the animal involuntarily engages in ACRs to maintain blood pressure and prevent further decreases of heart rate (Youmans et al., 1974). Due to the inability of BAT to thermoregulate, USVs are emitted as by-products of the ACR.

As noted earlier, psychopharmacological manipulations can alter USV levels, therefore it is possible that these cardiovascular mechanisms can be activated using various drugs. Interestingly, drugs that enhance USVs often affect the cardiovascular system. For instance, both the κ-opioid agonist U50,488 and the α_2-noradrenergic agonist clonidine decrease body temperature, heart rate, and blood pressure of rats (Gautret & Schmitt, 1985; Ledda, Mantelli, & Corti, 1985; Szabo, Bock, Nordheim, & Niederhoffer, 1999). Interestingly, κ-opioid and α_2-noradrenergic systems interact when modulating rabbit and guinea pig heart functioning. For example, stimulation of κ-opioid receptors, located on peripheral noradrenergic nerve terminals, inhibits the release of norepinephrine at the heart (Fuder, Buder, Riers, & Rothacher, 1986; Ledda et al., 1985; Starke, Schoffel, & Illes, 1985). Similarly, stimulation of presynaptic α_2-adrenoceptors in the periphery

also inhibits the release of norepinephrine at the heart (Korner et al., 1983; Van Zwieten, 1986). Inhibiting norepinephrine release by κ-opioid or α_2-adrenoceptor stimulation decreases heart rate (bradycardia) (Fuder et al., 1986; Van Zwieten, 1986). Centrally mediated cardiovascular responses can decrease heart rate and blood pressure as well. For instance, activation of κ-opioid receptors in hippocampal and hypothalamic regions produces bradycardia and hypotension (Wang & Ingenito, 1994). Based on the evidence presented, it is possible that U50,488 and clonidine increase USVs through either peripherally- or centrally-mediated cardiovascular mechanisms.

Summary and proposal. At present, USV production can be explained using either the distress model or the cardiovascular model. The distress model states that USVs are an emotionally-mediated behavior produced by rat pups when separated from their mother and littermates. These vocalizations are therefore emitted, either intentionally or unintentionally, to attract the mother for nurturance, protection, and assistance in thermoregulation. The cardiovascular model, however, argues that USVs are simply by-products of ACRs. Thus, ultrasound production is

considered to be a residual effect, as the young rat involuntarily engages in ACRs to drive venous blood back to the heart.

Drug-induced changes in USVs can be explained using both the distress and cardiovascular models. That is, U50,488 and clonidine may increase USVs by: a) directly affecting neuronal mechanisms mediating distress, or b) altering heart rate, blood pressure and body temperature. Therefore, my long-term goal is to determine the underlying mechanisms by which κ-opioid agonists and noradrenergic agonists produce vocalizations. As an initial step, I want to determine whether the κ-opioid and α_2-noradrenergic systems interact when mediating USV production.

κ-Opioid and α-noradrenergic systems

κ-Opioid and α-noradrenergic receptors are intimately involved in USV production in young rats. For instance, both the κ-opioid agonist U50,488 and the α_2-noradrenergic agonist clonidine enhance USVs of neonatal and preweanling rats (Carden et al., 1994; Hård et al., 1988; Kehoe & Boylan, 1994; Kehoe & Harris, 1989; Nazarian et al., 1999). It is currently not known whether stimulation of the κ-opioid system is necessary for α-noradrenergic-induced

USVs. As noted earlier, it is possible that these two systems interact either centrally or peripherally to enhance USVs. Stimulation of α-noradrenergic or κ-opioid receptors located on noradrenergic nerve terminals in the heart inhibits the release of norepinephrine. The reduced levels of norepinephrine cause bradycardia, which may then enhance USVs. However, it is also possible that κ-opioid and noradrenergic systems interact in the amygdala, striatum, or PAG to increase USVs. To date, the interaction of κ-opioid and α-noradrenergic system on the USVs of young rats has not been examined. Consequently, studying how these two systems interact is crucial for better understanding the underlying mechanisms responsible for USV production.

κ-Opioid receptors. Since the discovery of κ-opioid receptors in the early 1980's, three κ-opioid receptor subtypes have been characterized: κ_1, κ_2, and κ_3 (Cheng, Roques, Gacel, Huang, & Pasternak, 1992; Fowler & Fraser, 1994; Leslie & Laughlin, 1994; Unterwald, Knapp, & Zukin, 1991). The κ_1-opioid receptor subtype has a high affinity for the endogenous peptide dynorphin A and exogenous arylacetamide drugs such as U50,488 and U69,593 (Devlin &

Shoemaker, 1990; Unterwald et al., 1991). The κ_2-opioid receptor has a higher affinity for dynorphin A and exogenous drugs such as bremazocine (Underwald et al., 1991). Because of the limited understanding of κ_3-opioid receptors, the only drug that has specific affinity for this receptor subtype is naloxone benzoylhydrazone (Cheng et al., 1992).

κ_1-Opioid receptors are found in various brain regions, including the striatum, nucleus accumbens, olfactory tubercle, amygdala, central medial nucleus of the thalamus, ventral tagmental area, periaqueductal gray, and substantia nigra pars reticulata (Mansour, Burke, Pavlic, Akil, & Watson, 1996; Unterwald et al., 1991). In general, κ_2-opioid receptors are found in greater densities in the brain than κ_1-opioid receptors. κ_2-Opioid receptors have been localized in the striatum, nucleus accumbens, olfactory tubercle, claustrum, endopiriform nucleus, various thalamic regions, and inferior colliculus (Unterwald et al., 1991). The limited number of studies examining κ_3-opioid receptors suggest that they can be found in the thalamus, hypothalamic areas, hippocampus, striatum, and midbrain (Cheng et al., 1992). Localization of κ_3-

opioid receptors are based on homogenate receptor binding assays (Cheng et al., 1992), while κ_1-opioid and κ_2-opioid receptor localization is based on receptor autoradiography assays (Devlin & Shoemaker, 1990; Unterwald et al., 1991). Receptor autoradiography is more appropriate for studying regional differences in receptor topography than homogenate receptor binding assays (Feldman, Meyer, & Quenzer, 1997, pp. 31).

Behavioral effects of κ-opioid receptor stimulation in the adult rat. Stimulation of κ-opioid receptors produces antinociception in a variety of analgesia paradigms. For instance, the κ-opioid receptor agonist U50,488 increases radiant heat tail-flick latencies and hind paw withdrawal latencies in the formalin test, and decreases abdominal licking to visceral pain (Craft, Henley, Haaseth, Hruby, & Porreca, 1995; Idänpään-Heikkilä, Kalso, & Seppälä, 1994; McLaughlin, Tao, & Abood, 1995; Millan, Czlonkowski, Lipkowski, & Herz, 1989). Moreover, the κ-opioid antagonist *nor*-BNI attenuates U50,488-induced antinociception (Craft et al., 1995; McLaughlin et al., 1995; Millan et al., 1989). Therefore, these results demonstrate that U50,488-

induced antinociception is mediated by activation of κ-opioid receptors.

κ-Opioid receptor agonists produce a dose-dependent decrease in the locomotor activity of adult rats and mice (Jackson & Cooper, 1988; Leyton & Stewart, 1992; McLaughlin et al., 1995; Ukai & Kameyama, 1985; VonVoigtlander, Lahti, & Ludens, 1983). Thus, high doses (10 mg/kg) of U50,488 attenuate the locomotor activity of adult rats, while lower doses (1 mg/kg) of U50,488 have no effect (Jackson & Cooper, 1988; Ukai & Kameyama, 1985). U50,488's hypolocomotive effects are reversed by the κ-opioid receptor antagonist *nor*-BNI (Jones & Holtzman, 1992). In summary, it is evident that κ-opioid agonists reduce the locomotor activity of adult rodents via stimulation of κ-opioid receptors.

In addition to producing analgesia and affecting locomotor activity, activation of κ-opioid receptors appears to have some aversive properties. For example, stimulation of κ-opioid receptors using U50,488 or dynorphin derivatives (e.g., E-2078) results in conditioned place and taste aversions (Bals-Kubik, Herz, & Shippenberg, 1989; Mucha & Herz, 1985; Shippenberg & Herz, 1986; but see Crawford,

McDougall, Bolanos, Hall, & Berger, 1995). Moreover, κ-opioid receptor stimulation has been shown to be aversive when assessed in a self-administration paradigm (Di Chiara & Imperato, 1988b; Holtzman & Steinfels, 1994). Although speculative, U50,488-induced aversions and hypolocomotion may be due to κ-opioid-mediated reductions in extracellular dopamine in the nucleus accumbens and striatum (brain regions known to mediate both reward and locomotor activity) (Di Chiara & Imperato, 1988a; Maisonneuve, Archer & Glick, 1994; Spanagel, Almeida, Bartl, & Shippenberg, 1994).

Behavioral effects of κ-opioid receptor stimulation in the preweanling rat. Depending on the behavior, stimulation of κ-opioid receptors may produce adult-typical or adult-atypical effects in preweanling rats. Unlike adults, stimulation of κ-opioid receptors increases the locomotor activity of preweanling rats. For example, both systemic and intranigral injections of κ-opioid receptor agonists enhance the locomotor activity of 3-, 10-, and 17-day-old rats (Collins, Zavala, Nazarian, & McDougall, 2000; Duke, Meier, Bolanos, Crawford, & McDougall, 1997; Jackson & Kitchen, 1989; Kehoe & Boylan, 1994; McDougall, Rodarte-

Freeman, & Nazarian, 1999; McLaughlin et al., 1995). This increase in locomotion is blocked by systemic, as well as intranigral, injections of *nor*-BNI (Collins et al., 2000; McLaughlin et al., 1995), thus indicating that κ-opioid agonists produce their locomotor activating effects by stimulating κ-opioid receptors in the substantia nigra pars reticulata (Collins et al., 2000).

Similar to adults, preweanling rats given κ-opioid receptor agonists exhibit analgesic responses on tail-flick, hot-plate, formalin, and mechanical pain tests (Barr, Miya, & Paredes, 1992; Barr, Paredes, Erickson, & Zukin, 1986; Giordano & Barr, 1988; Kehoe & Boylan, 1994; McLaughlin et al., 1995; Nazarian et al., 1999). κ-Opioid-induced analgesia in the formalin test is blocked by the selective κ-opioid receptor antagonist *nor*-BNI (McLaughlin et al., 1995). Analgesia produced by stimulation of κ-opioid receptors is mediated through both spinal and superspinal sites (Barr et al., 1992; Goodwin, Wiedenmayer, & Barr, 1998).

Of course, U50,488 greatly enhances USVs in neonatal and preweanling rats (Barr et al., 1994; Carden, et al., 1994; Kehoe & Boylan, 1994; Nazarian et al., 1999).

U50,488-induced USVs may result from aversive properties of the drug, or κ-opioid receptors may directly mediate distress-induced vocalizations. These results indicate that preweanling rats have adult-typical analgesic and aversive reactions to κ-opioid agonists, while the mechanisms responsible for κ-opioid-mediated USVs and distress are not well understood.

α-Noradrenergic receptors (adrenoceptors). Knowing the neuroanatomical location and affinity of receptors is important when studying the functional role of receptor subtypes in the brain. Recent studies have categorized α-adrenoceptors into two subtypes: α_1 and α_2. α_1-Adrenoceptors are found primarily in the cerebral cortex, striatum, thalamus, hypothalamus, hippocampus, pons, medulla, cerebellum, and spinal cord (Wilson & Minneman, 1989). Moreover, α_1-adrenoceptors are characterized by having high affinities for agonist drugs such as cirazoline, methoxamine, and phenylephrine. These receptors also have high affinities for antagonists such as prazosin, WB-4101, and phenoxybenzamine (Ruffolo, Nichols, Stadel, & Hieble, 1991).

α_2-Adrenoceptors are found in somewhat different regions than α_1-adrenoceptors. For example, α_2-adrenoceptors are found in the locus coeruleus, hypothalamus, basolateral and central amygdala, thalamus, substantia nigra pars reticulata, dentate gyrus, striatum, CA1 pyramidal cells, and hippocampus (Wamsley, Alburges, Hunt, & Bylund, 1992). Noradrenergic agonists such as clonidine, α-methyl-norepinephrine, and UK-14,304 stimulate α_2-adrenoceptors, while yohimbine, idazoxan, and rauwolscine antagonize α_2-adrenoceptors (Ruffolo et al., 1991).

Behavioral effects of α-adrenoceptor stimulation in the adult and preweanling rat. Stimulation of α-adrenoceptors by clonidine (an α_2 preferring agonist) results in the suppression of various behaviors in adult rats. For instance, clonidine decreases the locomotor activity, wall climbing, and nociception of adult rats (Delini-Stula, Baumann, & Buch, 1979; Drew, Gower, & Marriott, 1977; Fielding, Spaulding, & Lal, 1981; Fielding et al., 1978; Reinstein & Isaacson, 1977; Smythe & Pappas, 1989). Moreover, stimulation of α_2-adrenoceptors by clonidine causes sedation, catalepsy, and suppresses

avoidance acquisition (Kostowski et al., 1981; Laverty & Taylor, 1969; Reinstein & Isaacson, 1977). The depressive effects of clonidine are due to the stimulation of central α_2-noradrenergic autoreceptors which attenuate the release of norepinephrine from nerve terminals (Delini-Stula et al., 1979; Drew, Grower, & Marriott, 1977). Clonidine does have some rewarding properties, because it produces conditioned place preferences in adult rats (Asin & Wirtshafter, 1985).

In contrast to adults, young rats show different behavioral reactions to adrenoceptor agonists. More specifically, stimulation of α_2-adrenoceptors enhances USVs, general motoric movement, wall climbing, antinociception, and decreases olfactory place preference of home cage odor (Blumberg et al., 1999; Hansen, 1993; Hård et al., 1988; Kehoe & Harris, 1989; Smythe & Pappas, 1989). Clonidine-induced USVs and locomotor activity are inhibited by yohimbine and idazoxan (α_2-receptor antagonists) in a dose-dependent manner (Hård et al., 1988; Kehoe & Harris, 1989; Nomura & Segawa, 1979). When considered together, it is evident that stimulation of α-adrenoceptors produces different behavioral effects in young pups than adult rats,

thus indicating that the noradrenergic system undergoes ontogenetic changes across the preweanling period.

Cardiovascular system

Effects of κ-opioid receptor stimulation on the cardiovascular system. Numerous studies have shown the presence of κ-opioid receptors in rat heart (Tai, Jin, Chan, & Wong, 1991; Zhang, Wang, Xia, & Wong, 1997; Zimlichman et al., 1996). Peripheral administration of endogenous and exogenous κ-opioid ligands (e.g., dynorphin (1-13) or U50,488) decreases heart rate (bradycardia) and blood pressure of adult rats (Gautret & Schmitt, 1985; Ledda et al., 1985; Pugsley, Penz, Walker, Wong, 1992a; 1992b; Pugsley, Saint, Penz, & Walker, 1993). Interestingly, κ-opioid receptor agonists produce bradycardia through two distinctly different mechanisms of action. At lower doses, U50,488 produces bradycardia by stimulating κ-opioid receptors in the heart; while at higher doses U50,488 produces bradycardia by blocking Na^+ and K^+ currents in heart muscles (Gautret & Schmitt, 1985; Pugsley et al., 1992a; Pugsley et al., 1993; Pugsley, Saint, & Walker, 1994; Wong, Lee, & Tai, 1990). When administered at lower doses, U50,488-induced bradycardia is blocked by the κ-

opioid receptor antagonist MR 2266, or the non-selective opioid antagonist naloxone (Wong et al., 1990). At higher doses, U50,488-induced bradycardia is not affected by MR 2266 or naloxone (Pugsley et al., 1992a). Therefore, it appears that κ-opioid-induced bradycardia is mediated by two different mechanisms in rat heart.

In addition to these peripherally mediated effects, heart rate and blood pressure can be affected by central administration of drugs. There are various brain regions that, when stimulated, can alter heart rate and blood pressure. For example, U50,488 infused into the hippocampus or hypothalamus produces bradycardia and hypotension; similar to the effects of peripherally administered κ-opioid agonists (Feuerstein & Faden, 1982; Wang & Ingenito, 1994).

Altogether, at least two possible mechanisms of action can account for κ-opioid mediated bradycardia and hypotension. Peripherally, κ-opioid receptor stimulation at the heart can produce bradycardia and hypotension. While, central activation of κ-opioid receptors in the hippocampus or hypothalamus can also decrease heart rate and blood pressure.

Effect of α-noradrenergic receptor stimulation on the cardiovascular system. The α-noradrenergic system is intimately involved in the normal functioning of the heart. Consequently, modulation of the α-noradrenergic system using agonist and antagonist drugs can significantly alter heart rate and blood pressure of the organism. For instance, stimulation of cardiac α_1-adrenoceptors by endogenous or exogenous ligands increases heart rate and blood pressure (Van Zwieten, Timmermans, & Van Brummelen, 1984; Van Zwieten, Van Meel, & Timmermans, 1982). In rat heart, α_1-adrenoceptors appear to be exclusively located on postsynaptic terminals (Wagner & Brodde, 1978).

Unlike α_1-adrenoceptors, α_2-adrenoceptors are located on presynaptic terminals in rat heart (Langer, 1981; Starke, 1977; Westfall, 1977). Stimulation of α_2-adrenoceptors by norepinephrine or clonidine inhibits norepinephrine release from presynaptic terminals (Korner et al., 1983; Van Zwieten, 1986). Therefore, stimulation of α_2-adrenoceptors reduces blood pressure and heart rate in rats by reducing norepinephrine levels at the heart (Korner et al., 1983; Szabo et al., 1999). In summary, stimulation

of postsynaptic α_1-adrenoceptors increases heart rate and blood pressure, while stimulation of presynaptic α_2-adrenoceptors decreases heart rate and blood pressure.

Overview

As previously mentioned, USV production has been explained by two distinctly different models: the distress model and the cardiovascular model. The distress model suggests that USVs are an emotionally-mediated reaction exhibited by young pups. This model is supported by findings showing that young rats emit USVs when separated from their dam and littermates. Pharmacological findings indicate that various drugs alter USV production. For instance, morphine, cocaine, and diazepam reduce vocalizations, while U50,488 and clonidine enhance vocalizations.

In opposition to the distress model, the cardiovascular model suggests that the cardiovascular system may be ultimately responsible for both U50,488- and clonidine-induced vocalizations. That is, alterations in the cardiovascular system due to isolation or drugs may result in the production of USVs. Pharmacological evidence indicates that both U50,488 and clonidine produce

bradycardia, hypotension, and hypothermia. These cardiovascular effects are important factors that can activate ACRs and, thus, produce USVs. Therefore, it is possible that isolation-induced and pharmacologically-induced USVs are due to cardiovascular mechanisms instead of emotionally-induced reactions such as distress.

Altogether, it appears that activating κ-opioid and noradrenergic systems enhances USV production of preweanling rats. This increase may be due to either emotional- or cardiovascular-mediated actions. Because κ-opioid and noradrenergic agonist drugs increase USVs, it appears that both the κ-opioid and noradrenergic neurotransmitter systems mediate USV production. It is uncertain, however, whether these two neurotransmitter systems interact to mediate USVs.

Thesis

The purpose of this thesis was to determine whether the κ-opioid and α_2-noradrenergic systems interact to mediate USV production of preweanling rats. The interaction of these systems was assessed by using the κ-opioid agonist U50,488, the κ-opioid antagonist *nor*-BNI, the

α_2-noradrenergic agonist clonidine, and the α_2-noradrenergic antagonist yohimbine.

Both USVs and line-crosses (a reliable measure of locomotor activity) were assessed in each experiment. In the first experiment, various doses of clonidine were administered in order to determine a dose of clonidine that would reliably produce USVs and line-crosses in young rats. In the second experiment, the interaction of κ-opioid and α_2-noradrenergic systems was assessed by administering *nor*-BNI in combination with U50,488 or clonidine. In the third experiment, the interaction of κ-opioid and α_2-noradrenergic systems was further assessed by administering yohimbine in combination with clonidine or U50,488.

It was originally hypothesized that the pattern of drug effects would differ depending on the dependent measure (i.e., USVs, locomotor activity, and rectal temperatures). When assessing USV production, it was predicted that: 1) clonidine and would produce a progressive dose-dependent increase in USVs; 2) *nor*-BNI would attenuate U50,488- and clonidine-induced USVs, and 3) yohimbine would attenuate clonidine- and U50,488-induced USVs. This pattern of results would indicate that κ-opioid

and α_2-noradrenergic systems synergistically interact when mediating USV production. The bases for these predictions were three-fold: first, stimulation of both κ-opioid and α_2-noradrenergic receptors enhances USV emissions; second, κ-opioid and α_2-noradrenergic systems are known to have extensive interactions in the periphery; and, third, USVs are suspected to be peripherally mediated. Because of the extensive peripheral interactions between κ-opioid and α_2-noradrenergic systems, it was predicted that these two neurotransmitter systems would interact to mediate USV production.

A different pattern of drug-induced effects was hypothesized for locomotor activity. It was predicted that: 1) clonidine would produce a progressive dose-dependent increase in line-crosses; 2) *nor*-BNI would attenuate U50,488-, but not clonidine-, induced line-crosses; and 3) yohimbine would attenuate clonidine-, but not U50,488-, induced line-crosses. The bases for these predictions were two-fold: first, it is doubtful whether κ-opioid and α_2-noradrenergic systems have extensive interconnections in brain regions known to mediate locomotor activity; and, second, κ-opioid and α_2-

noradrenergic receptor agonists are suspected of stimulating locomotor activity via central mechanisms (Andén et al., 1970; Collins et al., 2000; Żebrowska-Łupina, Prezegaliński, Słoniec, & Kleinrok, 1977). Because of the lack of known central interconnections, it is doubtful whether the κ-opioid and α_2-noradrenegic systems interact to mediate locomotor activity.

Hypotheses about drug-induced changes in rectal temperatures were based on previous findings. It was predicted that clonidine, but not U50,488, would reduce rectal temperatures of 11-day-old rats (Blumberg, Kreber, Sokoloff, & Kent, 2000; Nazarian et al., 1999). It was also predicted that yohimbine would attenuate clonidine-induced reductions in rectal temperatures.

Future Directions

This research project was the first step in determining whether: 1) the κ-opioid and α-noradrenergic systems interact when mediating USV production, 2) the κ-opioid and α-noradrenergic receptors mediating USVs are located centrally or peripherally, and 3) USVs are the result of distress or ACRs. In this study I directly answered the first question, that is, whether the κ-opioid

and α_2-noradrenergic systems interact when mediating USV production. In subsequent projects I hope to build on these results and provide answers for all three questions.

GENERAL METHODS

Subjects

Subjects were 175 rats of Sprague-Dawley descent (Harlan, Indianapolis, IN). Litters were culled to 10 pups on postnatal day (PND) 3. An approximately equal number of male and female rat pups were used in each experiment. All rats were tested on PND 11. Animals were housed with their dam and littermates prior to behavioral assessment. Rats were randomly assigned to groups with no more then one rat from each litter being placed in a particular group. To maintain litter size, previously tested animals were anesthetized with pentobarbital and placed back in the home cage (Carden, Bartot, & Hofer, 1993). The colony room was maintained at 22-24°C on a 12-hour light/dark cycle (lights on at 6 a.m.).

Apparatus

Ultrasonic vocalizations and locomotor activity were assessed in a Plexiglas chamber (19 × 19 × 19.5 cm) housed inside a heated incubator (34°C). Rectal temperatures were taken using a RET-3 rectal probe connected to a BAT-12 thermometer (Physitemp, Clifton, NJ). USVs were transduced using a Mini-3 ultrasonic detector (Ultrasound Advice,

London, England). An experimenter blind to treatment conditions counted vocalizations.

Drugs

(±)-*trans*-U-50,488 methanesulfonate, *nor*-binaltorphimine dihydrochloride (*nor*-BNI), clonidine hydrochloride, and yohimbine hydrochloride (Sigma, St. Louis, MO) were mixed in saline vehicle at a volume of 5 ml/kg. All injections were given intraperitoneally (ip).

Statistical Analysis

Analyses of variance (ANOVAs) were used to analyze USVs, line-crosses, and rectal temperatures. For all analyses, litter effects were controlled using within-litter statistical procedures (i.e., a within analysis with one value/condition/litter; Zorilla, 1997). In order to assess sex-induced behavioral effects, USVs, line-crosses, and body temperature data were reanalyzed with sex being included as a factor in the statistical analyses. None of these analyses resulted in statistically reliable effects, so analyses including sex as a factor were not presented in the text. Post hoc analysis of simple interactions and main effects were performed using Tukey HSD tests ($p <$

0.05). In these circumstances, Tukey tests were used to compare drug groups to saline controls.

EXPERIMENT 1

The first experiment was conducted to determine a dose of clonidine that reliably stimulates USVs in 11-day-old rats. It was predicted that clonidine would produce a dose-dependent increase in USVs.

Method

Eight litters (N = 40) of 11-day-old rats were injected with clonidine (0.05, 0.1, 0.25, or 0.5 mg/kg, ip) or saline and returned to the home cage for 15 min. Rats were then individually transported to the testing room and placed in the testing chamber. USVs and locomotor activity were assessed for 20 min. Rectal temperatures were taken immediately following testing.

In Experiment 1, separate one-way (agonist drug) ANOVAs were used to analyze USVs, line-crosses, and rectal temperatures. When appropriate, Tukey tests were used to assess differences between drug groups and saline controls.

Results

Ultrasonic Vocalizations. USVs data were analyzed using a one-way within-subjects ANOVA (see Appendix A). USVs of rats given clonidine are shown in Figure 1. Clonidine produced a dose-dependent increase in the USV production of 11-day-old rats [agonist drug main effect,

$F(4, 28) = 14.76$, $p < 0.001$]. Specifically, the two highest doses of clonidine (0.25 and 0.5 mg/kg) produced more USVs than saline [Tukey tests, $p < 0.05$].

Line-crosses. Line-cross data were analyzed using a one-way within-subjects ANOVA (see Appendix B). Line-crosses of young rats given clonidine are shown in Figure 2. When compared to the saline group, all doses of clonidine caused a significant reduction in line-crosses [agonist drug main effect, $F(4, 28) = 4.93$, $p < 0.01$, and Tukey tests, $p < 0.05$].

Rectal Temperatures. Rectal temperature data were analyzed using a one-way within-subjects ANOVA (see Appendix C). Rectal temperatures of rats given clonidine are shown in Figure 3. Rats treated with higher doses of clonidine (0.1, 0.25, or 0.5 mg/kg) had significantly lower rectal temperatures than saline-treated rat pups [agonist drug main effect, $F(4, 28) = 12.24$, $p < .001$, and Tukey tests, $p < 0.05$].

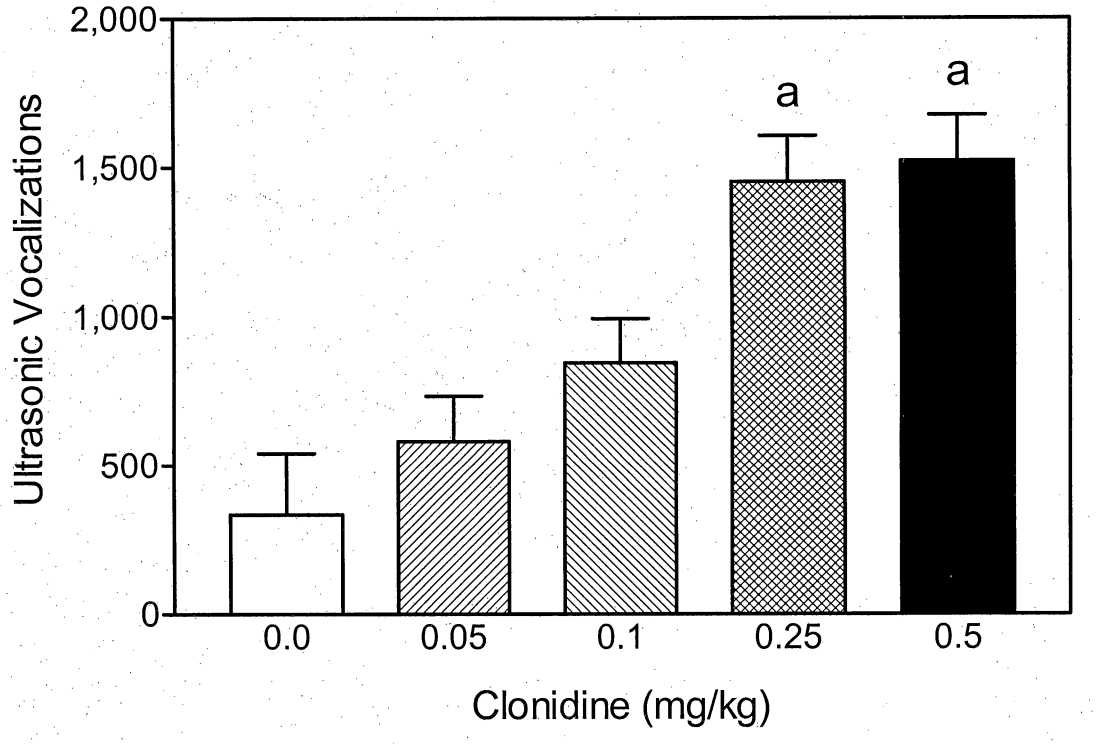

Figure 1. Mean ultrasonic vocalizations (±SEM) of 11-day-old rats (n = 8) administered saline or clonidine (0.05, 0.1, 0.25, or 0.5 mg/kg) 15 min prior to behavioral testing. [a]Significantly different from saline-treated rats.

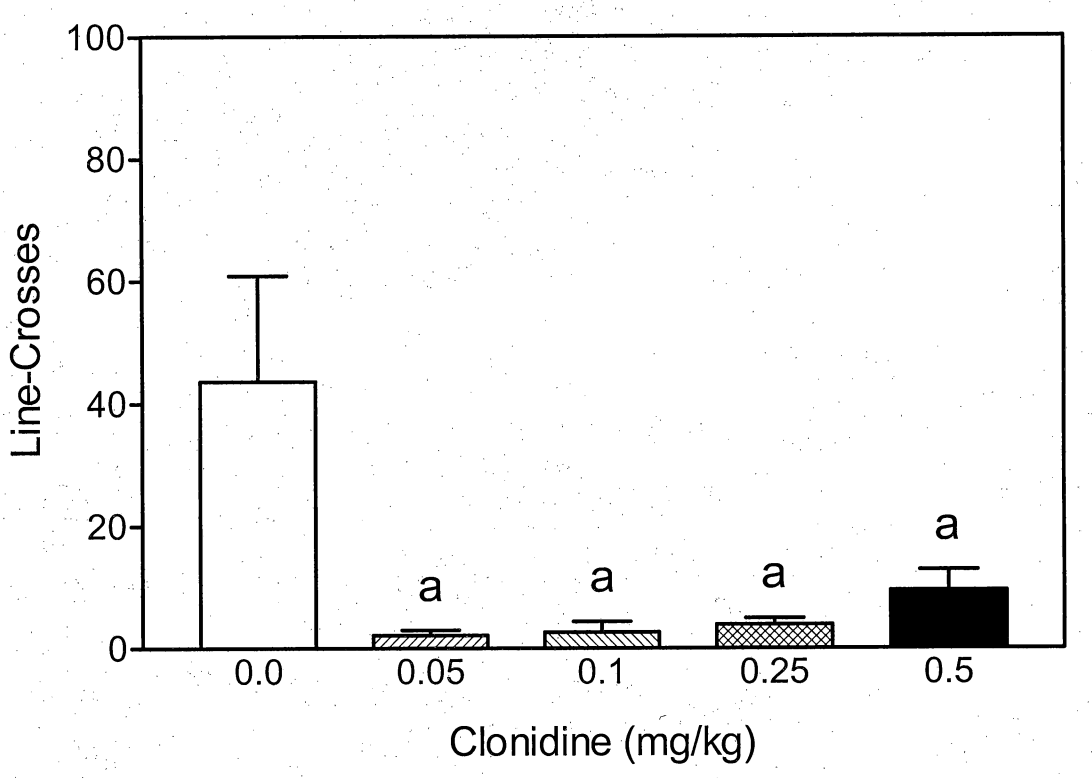

Figure 2. Mean line-crosses (±SEM) of 11-day-old rats (n = 8) administered saline or clonidine (0.05, 0.1, 0.25, or 0.5 mg/kg) 15 min prior to behavioral testing.
[a]Sgnificantly different from saline-treated rats.

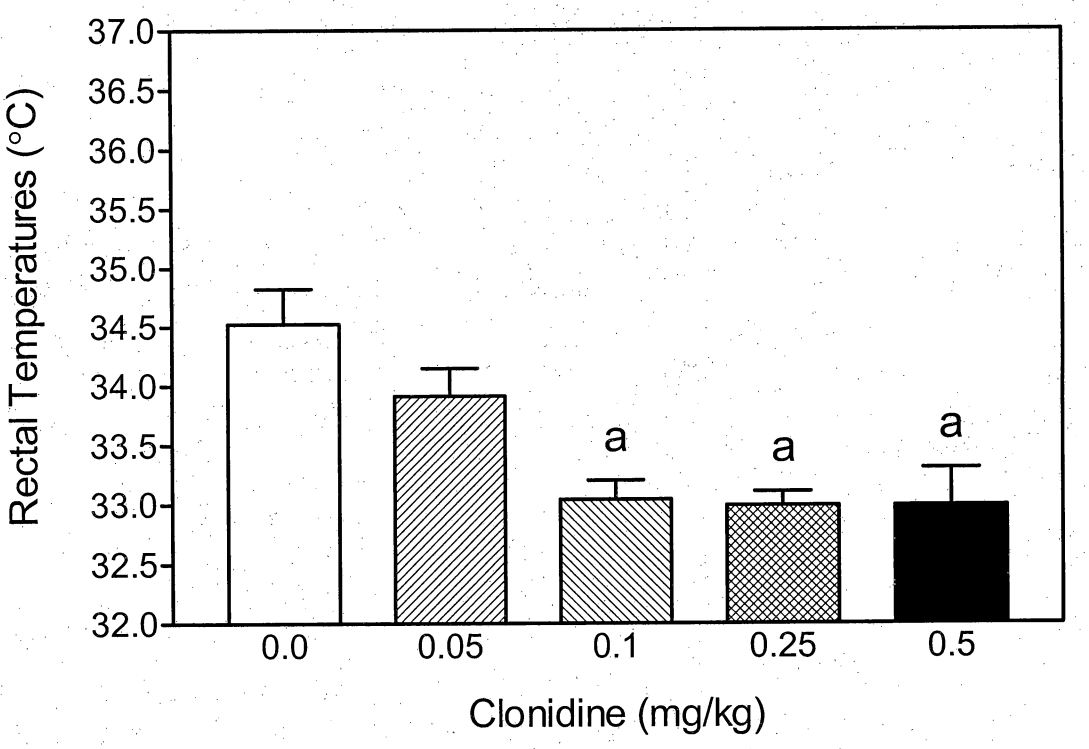

Figure 3. Mean rectal temperatures (±SEM) of 11-day-old rats (n = 8) administered saline or clonidine (0.05, 0.1, 0.25, or 0.5 mg/kg) 15 min prior to behavioral testing. Rectal temperatures were measured immediately following the 20 min behavioral testing session. [a]Significantly different from saline-treated rats.

EXPERIMENT 2

In the second experiment I assessed the potential interaction between the κ-opioid and α_2-noradrenergic systems. To do so, the κ-opioid antagonist *nor*-BNI was injected prior to treatment with the κ-opioid agonist U50,488 or the α-noradrenergic agonist clonidine. It was predicted that κ-opioid receptor blockade would attenuate both κ-opioid-mediated and α-noradrenergic-mediated USV production.

Method

Eight litters (N = 72) of 11-day-old rats were injected with *nor*-BNI (0, 5, or 10 mg/kg, ip) and placed in their home cage for 15 min. Rat pups were then injected with saline, U50,488 (2.5 mg/kg, ip) or clonidine (0.25 mg/kg, ip). After an additional 15 min, USVs and locomotor activity were measured in the testing chamber for 20 min (divided into four 5-min time blocks). Time blocks were used to assess whether drug-induced effects varied as a function of time. Rectal temperatures were taken immediately after testing. Rat pups were then placed in a room separate from their dam and littermates.

In Experiment 2, a 3 × 3 × 4 (antagonist drug × agonist drug × time) within-subjects repeated measures ANOVA was used to analyze USVs. Line-crosses and rectal temperatures were analyzed using 3 × 3 (antagonist drug × agonist drug) within-subjects ANOVAs.

Results

Ultrasonic Vocalizations. USV data were analyzed using a 3 × 3 × 4 within-subjects repeated measures ANOVA (see Appendix D). USVs of rats given *nor*-BNI and U50,488 or clonidine are shown in Figure 4. Overall, rats given U50,488 or clonidine emitted more USVs than saline-treated rats across all time blocks (all three graphs) [agonist drug × time interaction, $F(6, 42) = 6.20$, $p < 0.001$, and Tukey tests, $p < 0.05$]. *nor*-BNI alone had no effect on USV production (upper graph); however, both doses of *nor*-BNI (5 and 10 mg/kg) attenuated the U50,488-induced USV production of 11-day-old rats (middle graph) [antagonist drug × agonist drug interaction, $F(4, 28) = 20.87$, $p < 0.001$, and Tukey tests, $p < 0.05$]. *nor*-BNI did not decrease clonidine-induced USV production (bottom graph).

Line-crosses. Line-cross data were analyzed using a 3 × 3 within-subjects ANOVA (see Appendix E). Line-crosses of

rats given *nor*-BNI and U50,488 or clonidine are shown in Figure 5. U50,488-treated rats had more line-crosses than their saline controls, while clonidine had no effect [agonist drug main effect, $F(2, 14) = 68.44$, $p < 0.001$, and Tukey tests, $p < 0.05$]. *nor*-BNI (5 or 10 mg/kg) attenuated U50,488-induced line-crosses in a dose-dependent manner, whereas *nor*-BNI did not affect line-crosses of saline- or clonidine-treated rats [antagonist drug × agonist drug interaction, $F(4, 28) = 11.84$, $p < 0.001$, and Tukey tests, $p < 0.05$]. Curiously, the number of line-crosses exhibited by saline-treated rats ($M = 3.50$) were less than in Experiment 1 ($M = 43.62$). This was most likely due to handling stress caused by multiple injections.

Rectal Temperatures. Rectal temperature data were analyzed using a 3 × 3 within-subjects ANOVA (see Appendix F). Rectal temperatures of rats given *nor*-BNI and U50,488 or clonidine are shown in Figure 6. Rats given clonidine had lower rectal temperatures than saline controls [agonist drug main effect, $F(2, 14) = 30.21$, $p < 0.001$, and Tukey tests, $p < 0.05$]. When given alone, neither U50,488 nor *nor*-BNI affected rectal temperatures. *nor*-BNI did not alter clonidine-induced reductions in rectal temperatures.

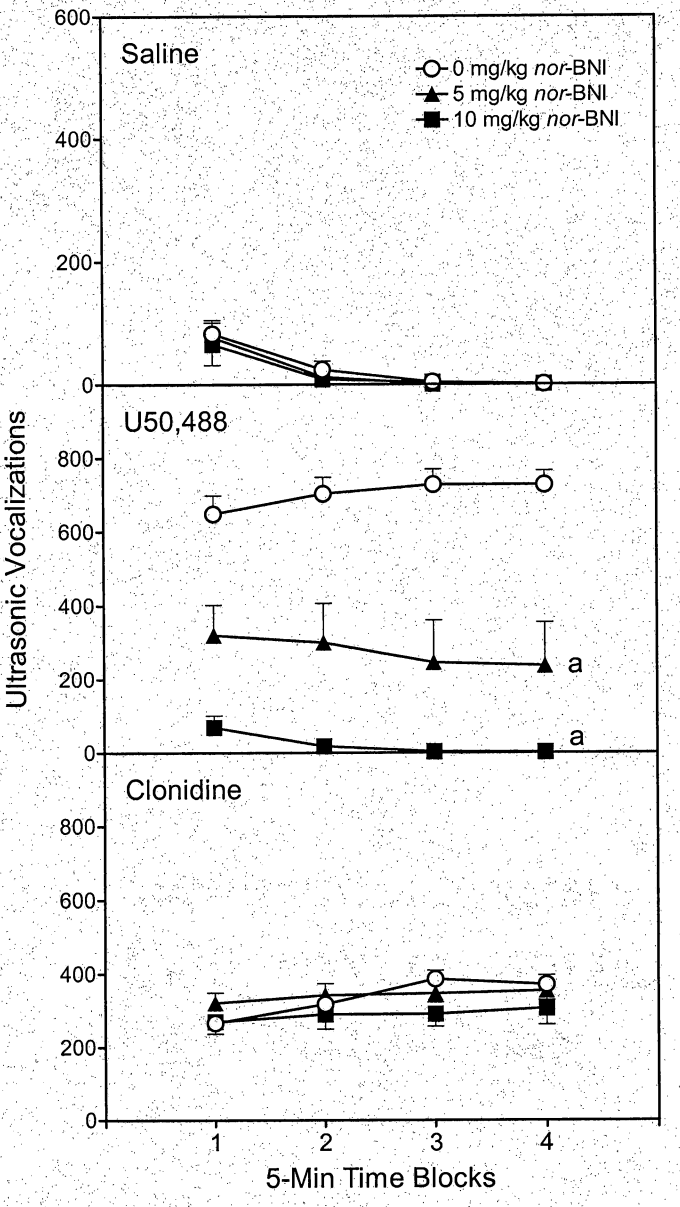

Figure 4. Mean ultrasonic vocalizations (±SEM) of 11-day-old rats (n = 8) administered *nor*-BNI (0, 5, or 10 mg/kg) 30 min prior to behavioral testing and saline, U50,488 (2.5 mg/kg) or clonidine (0.25 mg/kg) 15 min prior to behavioral testing. [a]Significantly different from rats given U50,488 and 0 mg/kg *nor*-BNI collapsed across all time blocks (open circles).

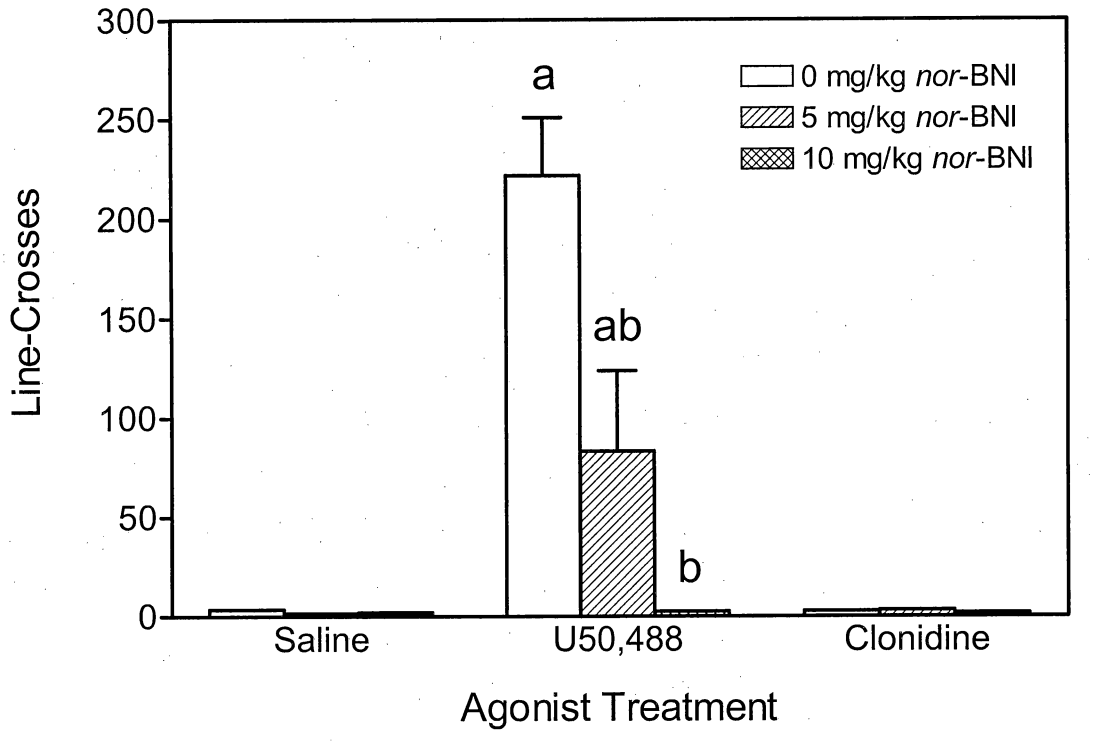

Figure 5. Mean line-crosses (±SEM) of 11-day-old rats (n = 8) administered *nor*-BNI (0, 5, or 10 mg/kg) 30 min prior to behavioral testing and saline, U50,488 (2.5 mg/kg) or clonidine (0.25 mg/kg) 15 min prior to behavioral testing. [a]Significantly different from similarly treated rats given saline; [b]Significantly different from rats given U50,488 and 0 mg/kg *nor*-BNI.

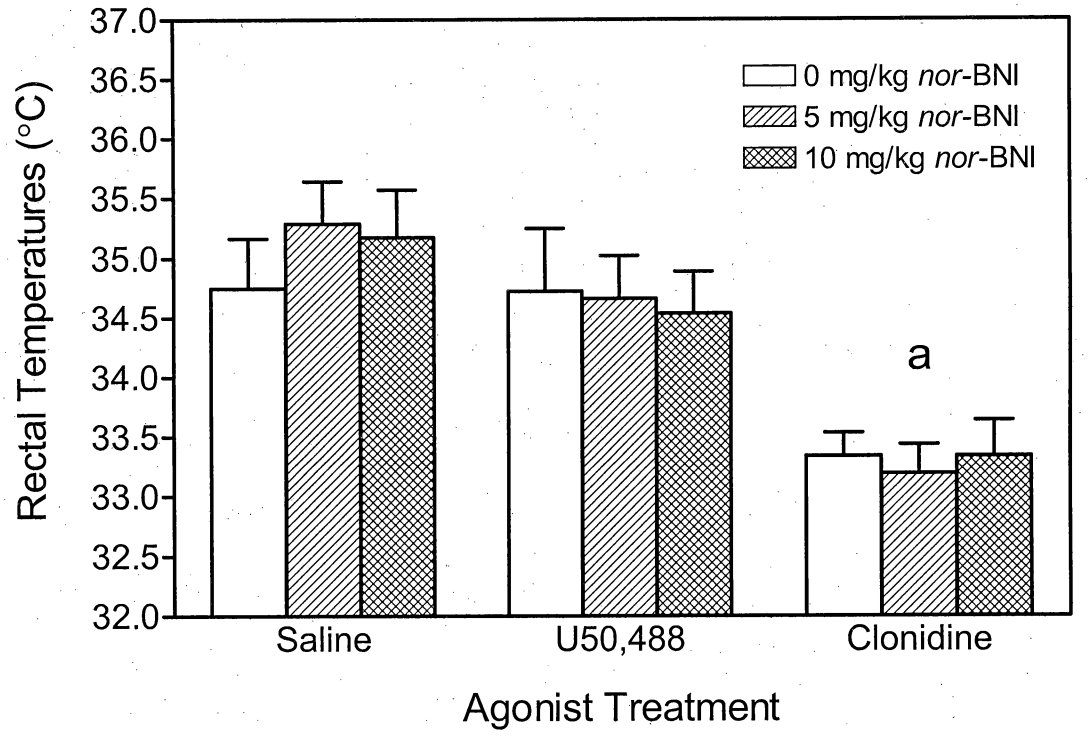

Figure 6. Mean rectal temperatures (±SEM) of 11-day-old rats (n = 8) administered *nor*-BNI (0, 5, or 10 mg/kg) 30 min prior to behavioral testing and saline, U50,488 (2.5 mg/kg), or clonidine (0.25 mg/kg) 15 min prior to behavioral testing. Rectal temperatures were measured immediately following the 20 min behavioral testing session. [a]Significantly different from saline-treated rats collapsed across agonist treatment.

EXPERIMENT 3

It was expected that *nor*-BNI would attenuate U50,488- and clonidine-induced USVs. Therefore, in the third experiment, the interaction of κ-opioid and α_2-noradrenergic systems were further investigated by injecting yohimbine in combination with clonidine or U50,488. I predicted that yohimbine would cause a dose-dependent decrease in the number of U50,488- and clonidine-induced USVs.

Method

Eight litters (N = 72) of 11-day-old rats were injected with yohimbine (0.0, 0.5, or 1.0 mg/kg, ip) and placed in their home cage for 15 min. Rat pups were then injected with saline, U50,488 (2.5 mg/kg, ip) or clonidine (0.25 mg/kg, ip). After an additional 15 min, USVs and locomotor activity were measured in the testing chamber for 20 min (divided into four 5-min time blocks). Rectal temperatures were taken immediately after testing. Rat pups were then placed in a room separate from their dam and littermates.

To analyze USVs, a 3 × 3 × 4 (antagonist drug × agonist drug × time) within-subjects repeated measures ANOVA was used. Line-crosses and rectal temperatures were analyzed

using 3 × 3 (antagonist drug × agonist drug) within-subjects ANOVAs.

Results

Ultrasonic Vocalizations. USV data were analyzed using a 3 × 3 × 4 within-subjects repeated measures ANOVA (see Appendix G). USVs of rats given yohimbine and U50,488 or clonidine are shown in Figure 7. Rats treated with U50,488 or clonidine produced more USVs than saline-treated rats [agonist drug main effect, $F(2, 12) = 49.71$, $p < 0.001$, and Tukey tests, $p < 0.05$]. Yohimbine (0.5 or 1.0 mg/kg) decreased USV emissions of saline-treated rats on time blocks 1 and 2 (upper graph) [antagonist drug × agonist drug × time interaction, $F(12, 72) = 2.94$, $p < 0.01$, and Tukey tests, $p < 0.05$]. Yohimbine also attenuated U50,488-induced USV production in a dose-dependent manner. Specifically, 0.5 mg/kg yohimbine decreased U50,488-induced USV production on time blocks 1, 3, and 4 [Tukey tests, $p < 0.05$]; whereas, 1.0 mg/kg yohimbine decreased U50,488-induced USVs on all time blocks (middle graph) [Tukey tests, $p < 0.05$]. Both doses of yohimbine (0.5 or 1.0 mg/kg) decreased clonidine-induced USV production on all time blocks (bottom graph) [Tukey tests, $p < 0.05$].

Line-crosses. Line-cross data were analyzed using a 3 × 3 within-subjects ANOVA (see Appendix H). Line-crosses of rats given yohimbine and U50,488 or clonidine are shown in Figure 8. Rats given U50,488, but not clonidine, had more line-crosses than saline-treated rats [Tukey tests, $p < 0.05$]. Interestingly, both doses of yohimbine (0.5 and 1.0 mg/kg) attenuated U50,488-induced line-crosses [antagonist drug × agonist drug interaction, $F(4, 24) = 9.55$, $p < 0.001$, and Tukey tests, $p < 0.05$]. Yohimbine did not affect line-crosses of saline- or clonidine-treated rats. The total number of line-crosses produced by saline-treated rats was consistent with Experiment 2.

Rectal Temperatures. Rectal temperature data were analyzed using a 3 × 3 within-subjects ANOVA (see Appendix I). Rectal temperatures of rats given yohimbine and U50,488 or clonidine are shown in Figure 9. Once again, clonidine-treated rats had lower rectal temperatures than saline- or U50,488-treated rats [agonist drug main effect, $F(2, 12) = 27.00$, $p < 0.001$, and Tukey tests, $p < 0.05$]. Overall, yohimbine did not affect the rectal temperatures of rat pups.

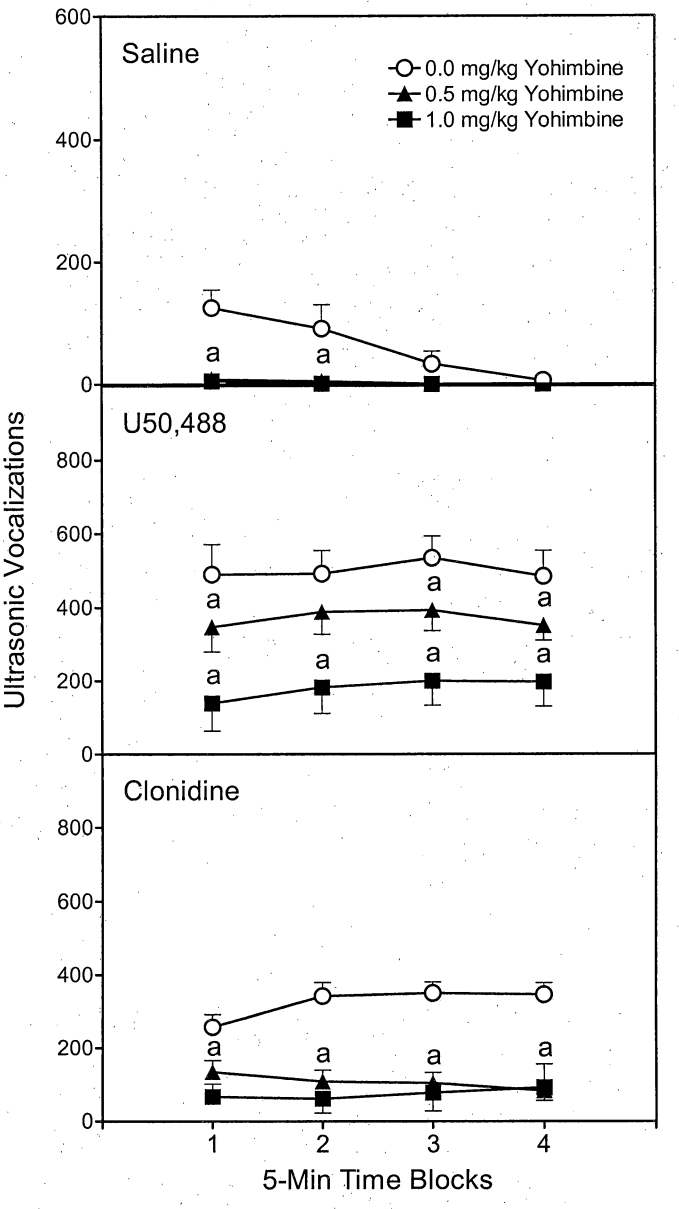

Figure 7. Mean ultrasonic vocalizations (±SEM) of 11-day-old rats (n = 7) administered yohimbine (0.0, 0.5, or 1.0 mg/kg) 30 min prior to behavioral testing and saline, U50,488 (2.5 mg/kg), or clonidine (0.25 mg/kg) 15 min prior to behavioral testing. [a]Significantly different from rats given 0.0 mg/kg yohimbine (open circles).

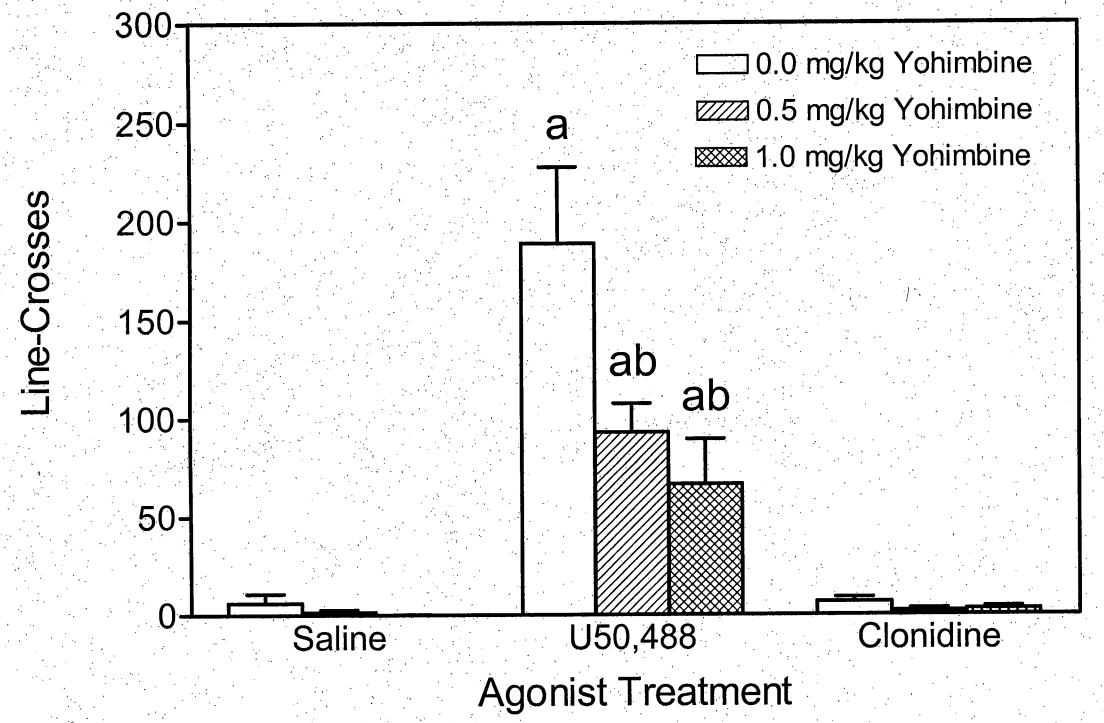

Figure 8. Mean line-crosses (±SEM) of 11-day-old rats (n = 7) administered yohimbine (0.0, 0.5, or 1.0 mg/kg) 30 min prior to behavioral testing and saline, U50,488 (2.5 mg/kg) or clonidine (0.25 mg/kg) 15 min prior to behavioral testing. [a]Significantly different from similarly treated rats given saline; [b]Significantly different from rats administered U50,488 and 0.0 mg/kg yohimbine.

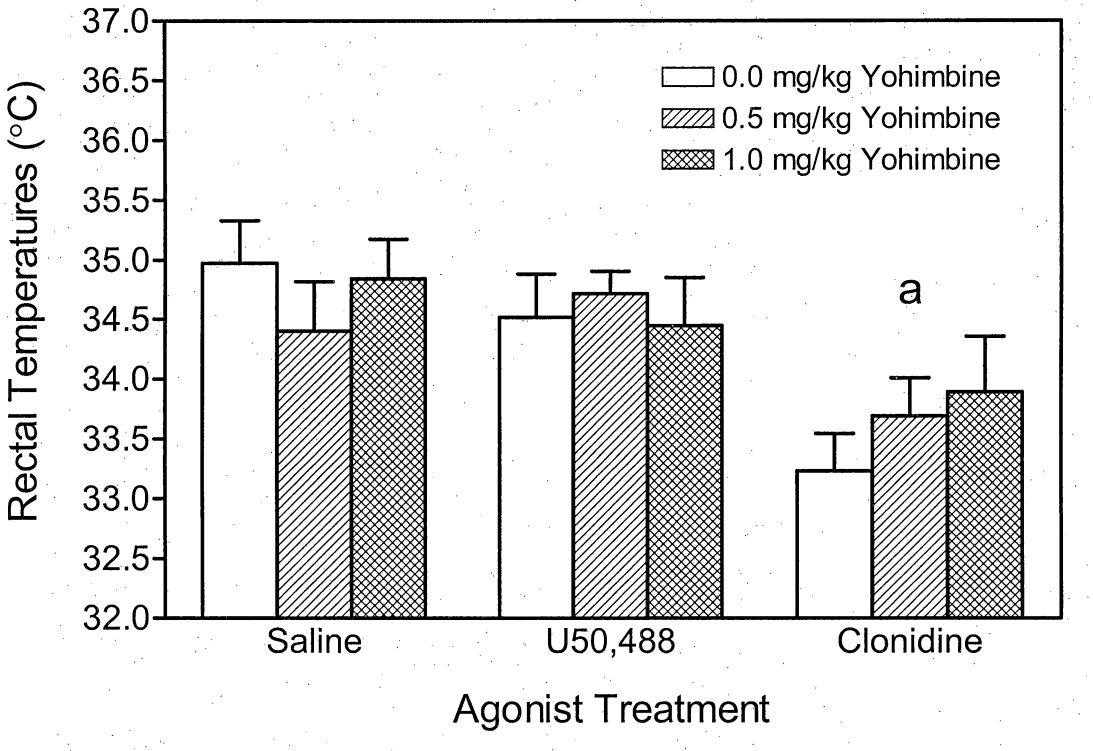

Figure 9. Mean rectal temperatures (±SEM) of 11-day-old rats (n = 7) administered yohimbine (0.0, 0.5, or 1.0 mg/kg) 30 min prior to behavioral testing and saline, U50,488 (2.5 mg/kg), or clonidine (0.25 mg/kg) 15 min prior to behavioral testing. Rectal temperatures were measured immediately following the 20 min behavioral testing session. [a]Significantly different from saline-treated rats collapsed across agonist treatment.

DISCUSSION

The purpose of the present study was to assess whether the κ-opioid and the α_2-noradrenergic systems interact when mediating USV production and locomotor activity of preweanling rats. Similar to previous studies, saline-treated rats emitted a moderate number of isolation-induced USVs that decreased as the testing session progressed (Blumberg, Efimova, & Alberts, 1992; Kehoe & Harris, 1989). As predicted, clonidine increased the USV production of 11-day-old rats in a dose-dependent manner (see Blumberg, Kreber et al., 2000; Hansen, 1993; Hård et al., 1988; Kehoe & Harris, 1989). Also, rat pups treated with U50,488 emitted more USVs than saline-treated rats (see Barr et al., 1994; Carden et al., 1994; Kehoe & Boylan, 1994; Nazarian et al., 1999).

The interaction of κ-opioid and α_2-noradrenergic systems was examined in two ways. First, by assessing the effects of the κ-opioid antagonist *nor*-BNI on U50,488- and clonidine-induced USV production. Second, by assessing the effects of the α_2-noradrenergic antagonist yohimbine on U50,488- and clonidine-induced USV production. In contrast to my initial prediction, *nor*-BNI decreased U50,488-, but

not clonidine-, induced USVs. Yohimbine, on the other hand, decreased both U50,488- and clonidine-induced USVs. Therefore, it appears that the κ-opioid and α_2-noradrenergic systems interact when mediating USV production, but in a more complex manner than originally hypothesized.

The κ-opioid agonist affected locomotor activity in a consistent manner, because rat pups treated with U50,488 had more line-crosses than saline-treated rats (see Experiments 2 and 3). In contrast, clonidine's effects on locomotor activity varied according to experiment. More specifically, clonidine reduced the line-crosses of preweanling rats in Experiment 1; whereas, clonidine did not significantly affect line-crosses in Experiments 2 and 3. The inability of clonidine to decrease line-crosses in the latter two experiments was probably due to a "basement effect", as the saline controls exhibited minimal line-crosses (M = 4.32) in Experiments 2 and 3 (i.e., a clonidine-induced reduction in line-crosses was impossible to detect). Both *nor*-BNI and yohimbine depressed U50,488-induced locomotor activity, while neither antagonist affected the locomotor activity of saline- or clonidine-treated rats.

Rectal temperatures were differentially affected by the various drugs. Clonidine consistently reduced rectal temperatures of rat pups in all three experiments. On the other hand, U50,488 did not affect rectal temperatures. Neither antagonist was able to block the clonidine-induced reduction in rectal temperatures.

USV production: Role of the α_2-noradrenergic system. As just mentioned, clonidine enhanced the USV production of 11-day-old rats. Clonidine-induced USVs can be explained through at least three different mechanisms. First, clonidine may stimulate α_2-adrenoceptors in brain regions involved with USV production. More specifically, clonidine may induce USV production by stimulating α_2-adrenoceptors in brain regions involved with affective behaviors, such as the amygdala, striatum, nucleus accumbens, and periaqueductal gray (PAG).

Second, clonidine may indirectly enhance USV production by affecting central pathways mediating cardiovascular functioning. It has been previously shown that clonidine stimulates α_2-adrenoceptors in the medulla or other brain stem nuclei involved in cardiovascular functioning (Van Zwieten, 1986; 1996). This clonidine-

induced stimulation may enhance USV production by decreasing heart rate and blood pressure.

Third, clonidine may increase USV production by directly stimulating presynaptic α_2-adrenoceptors on nerve terminals synapsing the heart. Direct stimulation of presynaptic α_2-adrenoceptors inhibits the release of norepinephrine and decreases heart rate and blood pressure (Drew, 1976; Misu, Fujie, & Kubo, 1982). Altogether, it appears that clonidine may induce USVs by: 1) producing a state of emotional distress in the rat pup that results in USV emissions, 2) modulating circuitry in the brain stem involved with heart functioning, or 3) directly acting on peripheral noradrenergic neurons that decrease heart rate and, thus, increase USVs.

USV production: Role of the κ-opioid system. Similar to previous studies, U50,488 increased the USV production of 11-day-old rats (Barr et al., 1994; Carden et al., 1994; Kehoe & Boylan, 1994; Nazarian et al., 1999). One possibility is that U50,488 stimulates USV production by activating κ-opioid receptors in the striatum, nucleus accumbens, amygdala, or PAG (i.e., brain regions known to be involved in affective behaviors). Alternatively,

U50,488 may activate κ-opioid receptors in brain regions involved in cardiovascular functioning. For instance, stimulation of κ-opioid receptors in the hippocampus, hypothalamus, and medulla decreases the heart rate and blood pressure of adult rats (Feuerstein & Faden, 1982; Hassen, Feuerstein, & Faden, 1984; Wang & Ingenito, 1994). The decreased heart rate could, in turn, be responsible for the increased USV emissions.

It is also possible that activating peripheral κ-opioid receptors may enhance USV emissions of rat pups. Stimulation of κ-opioid receptors, located on noradrenergic nerve terminals at the heart, decreases heart rate and blood pressure (Fuder et al., 1986; Ledda et al., 1985; Starke et al., 1985). Hence, the decrease in heart rate and blood pressure may increase USV production. Therefore, similar to clonidine, U50,488 may enhance USVs by: 1) producing a state of emotional distress by acting on brain regions involved in affective behaviors, 2) modulating brain circuitry concerned with heart functioning, or 3) directly inhibiting the release of norepinephrine at the heart. Taken together, it is possible that U50,488 and clonidine may produce USVs through similar mechanisms.

Role of the κ-opioid system on α_2-noradrenergic-mediated USV production. As noted earlier, the purpose of this study was to assess the interaction between the κ-opioid and the α_2-noradrenergic systems. The present study showed that *nor*-BNI attenuated U50,488-induced USV production of 11-day-old rats in a dose-dependent manner. On the other hand, *nor*-BNI (a κ-opioid antagonist) did not reduce the USVs of rats given clonidine (a α_2-noradrenergic agonist). Thus, results from this particular experiment suggest that the κ-opioid system does not modulate α_2-noradrenergic-mediated USV production.

Role of the α_2-noradrenergic system on κ-opioid mediated USV production. Other evidence suggests that the κ-opioid and α_2-noradrenergic systems interact when mediating USV production. Specifically, it appears that the α_2-noradrenergic system modulates κ-opioid-mediated USV emissions, as yohimbine decreased both U50,488- and clonidine-induced USV production in a dose-dependent manner. Yohimbine also reduced the USVs of saline-treated rats.

Based on this pattern of results, it appears that the α_2-noradrenergic system modulates κ-opioid-mediated USV production. Conversely, manipulation of the κ-opioid system had no effect on α_2-noradrenergic-mediated USVs. Therefore, the κ-opioid and α_2-noradrenergic systems seem to interact in a unidirectional manner. The most parsimonious explanation is that the κ-opioid receptors modulating USV production are located "up-stream" from the critical α_2-noradrenergic neurons. For instance, the κ-opioid system may mediate USV production through higher brain centers (e.g., the striatum or PAG), while the α_2-noradrenergic system may mediate USV production through brain stem areas. Due to this unidirectional arrangement, the α_2-noradrenergic system would be able to alter κ-opioid-mediated USV production, but the reverse would not be true.

The interaction between κ-opioid and α_2-noradrenergic systems on locomotor activity. In the present study, clonidine-treated 11-day-old rats had fewer line-crosses than saline controls. This result is somewhat curious because a number of studies have reported that clonidine enhances locomotor activity of rat pups (Kehoe & Harris,

1989; Nomura & Segawa, 1979; Pappas & Walsh, 1983; Reinstein & Issacson, 1977; Smythe & Pappas, 1989). This inconsistency is most likely due to the method of assessing locomotor activity. Studies that operationally define locomotor activity in terms of line-crosses (a direct measure of forward locomotion) typically report that clonidine reduces locomotion (Hansen, 1993; present study); whereas, studies that operationally define locomotor activity in terms of general motoric movement (i.e., by collapsing such measures as forward locomotion, paddling, circling, and wall climbing) find that clonidine enhances movement (Kehoe & Harris, 1989; Nomura & Segawa, 1979; Pappas & Walsh, 1983; Reinstein & Issacson, 1977; Smythe & Pappas, 1989). Consequently, it is likely that clonidine reduces the locomotor activity of preweanling rats, while increasing general motoric movement.

Stimulation of κ-opioid receptors produces a paradoxical increase in the locomotor activity of rat pups. That is, κ-opioid agonists (e.g., U50,488 and enadoline) decrease locomotor activity of adult rats, while increasing the locomotor activity of preweanling rats (Carden et al., 1994; Crawford et al., 1995; Kehoe & Boylan 1994; McDougall

et al., 1999; McLaughlin et al., 1995; VonVoigtlander et al., 1983). Consistent with past studies, U50,488 produced a *nor*-BNI reversible enhancement in the locomotor activity of 11-day-old rats (see also Collins et al., 2000; McLaughlin et al., 1995).

An unexpected finding was that yohimbine reduced U50,488-induced locomotor activity. Although not previously shown, it is possible that α_2-noradrenergic and κ-opioid systems interact to decrease U50,488-induced locomotor activity. This suggestion, however, requires further investigation. An alternative possibility is that yohimbine may decrease U50,488-induced locomotor activity by altering dopamine system functioning. Evidence for this possibility is three-fold. First, yohimbine antagonizes dopamine D_2-like receptors (Heal et al., 1987; Scatton, Zivkovic, & Dedek, 1980). Second, yohimbine attenuates amphetamine- and apomorphine-induced locomotor activity of adult rats (Heal et al., 1987; Luttinger & Durivage, 1986). Importantly, the doses of yohimbine (1 and 3 mg/kg) found to attenuate amphetamine- and apomorphine-induced locomotor activity were similar to those used in the present study. Third, dopamine antagonists attenuate U50,488-induced line-

crosses in rat pups (Duke et al., 1997; Nazarian et al., 1999). It should be noted that yohimbine does not reduce USV production by blocking D_2-like receptors because dopamine antagonists have no effect on USV production of rat pups (Dastur, McGregor, & Brown, 1999; Nazarian et al., 1999).

The role of κ-opioid and α_2-noradrenergic systems on rectal temperatures. Consistent with past studies, clonidine reduced rectal temperatures of 11-day-old rats (Hård et al., 1988). On the other hand, U50,488 did not reduce rectal temperatures. This may be due to the low dose (2.5 mg/kg) of U50,488 used in this study, because higher doses of U50,488 reduce rectal and axillary temperatures of rat pups (Carden et al., 1993; Nazarian et al., 1999). Interestingly, neither *nor*-BNI nor yohimbine had any effect on the clonidine-induced reduction of rectal temperatures. When considered together, these findings suggest that U50,488- and clonidine-induced USV production is independent of drug-induced changes in body temperature.

Conclusion. Overall, the results of the present study demonstrate that the κ-opioid and α_2-noradrenergic systems interact when mediating USV production. This study does

not conclusively resolve whether USV production is a central or peripheral effect, or whether USVs are the result of distress or a cardiovascular mechanism.

By combining both current and previous findings, it is possible to suggest a number of mechanisms that may be responsible for USV production. Although speculative, USV production may be a distress response mediated by brain regions involved in both affective and cardiovascular functioning. Specifically, in adult rats, distress can be induced by various procedures (e.g., restraint, shock, and tail-pinch), all of which increase norepinephrine levels in the amygdala, basal ganglia, hippocampus, hypothalamus, pons, and medulla (Quirarte, Galvez, Roozendaal, & McGaugh, 1998; M. Tanaka et al., 1983; T. Tanaka et al., 1991). These brain regions are involved in both affective (amygdala and basal ganglia) and cardiovascular (hippocampus, hypothalamus, pons, and medulla) functioning. It is possible that young rats may show similar increases in distress-induced norepinephrine levels. If this is true, then it may explain the underlying mechanisms responsible for USV production. That is, distress may increase norepinephrine levels in brain regions involved in affective and cardiovascular functioning. The increase in

norepinephrine levels may decrease blood pressure and heart rate and provoke USV emissions.

It is clear that USVs can be produced by distressing the rat pup or by independently altering the cardiovascular system. However, one should consider that both mechanisms may be jointly involved in producing USVs. That is, when an organism is crying, yelling, sneezing, or laughing, physiological mechanisms are involved that cause air to be exhaled from the larynx. Nonetheless, affect is inherently involved because voluntary actions (e.g., laughing or crying), similar to involuntary actions (e.g., sneezing), also activate the same physiological mechanisms (i.e., air exhaled from the larynx). When considering the adult and pup distress literature together, it appears that isolation-induced USV production is an emotionally-mediated behavior that requires the participation of brain regions involved in both affective and cardiovascular functioning.

Synopsis. The present study was a first step in trying to better understand the underlying mechanisms responsible for USV production. The function of USVs has been of great dispute in recent years (Blumberg, Sokoloff et al., 2000; Hofer & Shair, 1993). According to the distress model, USVs are interpreted to be an emotionally-

mediated behavior produced by rat pups in order to gain the attention of the dam for nurturance, protection, and thermoregulation (Bell, 1979; Smotherman et al., 1974). This model interprets USVs as a distress call emitted by rat pups, which can be modulated by various pharmacological agents known to alter distress and anxiety. For instance, drugs that reduce USV production are considered to be rewarding (e.g., cocaine and morphine) or anxiety reducing (e.g., diazepam), while drugs that enhance USVs (e.g., U50,488) are considered to be aversive or anxiogenic (Carden et al., 1990; 1994; Insel et al., 1986; Kehoe & Boylan, 1992). Therefore, according to the distress model, USVs are emotionally-mediated voluntary behaviors that are produced by rat pups when distressed.

In direct opposition, the cardiovascular model suggests that USVs are simply by-products of abdominal compression reactions (ACR). It is believed that rat pups produce USVs in order to maintain their normal heart rate, blood pressure, and body temperature when separated from the dam (Blumberg et al., 1999). The cardiovascular model postulates that drugs that enhance or reduce USV production either directly or indirectly modulate cardiovascular functioning through either central or peripheral mechanisms

(Blumberg, Kreber et al., 2000). Thus, drugs that cause a decrease in heart rate and blood pressure enhance USV production. On the other hand, drugs that increase heart rate and blood pressure reduce USV production. Taken together, proponents of the cardiovascular model discount the distress model and argue that USVs are a physiological response with no affective cause.

In the present study, the κ-opioid agonist U50,488, and the α_2-noradrenergic agonist clonidine independently enhanced USV production. I attempted to attenuate U50,488- and clonidine-induced USV production by using the κ-opioid antagonist *nor*-BNI and the α_2-noradrenergic antagonist yohimbine. By doing so, I was interested in learning whether the κ-opioid and the α_2-noradrenergic systems interact when mediating USV production. Results showed that the κ-opioid and the α_2-noradrenergic systems interact in a very specific manner. The findings demonstrate that U50,488-induced USV production was attenuated by *nor*-BNI and yohimbine, while clonidine-induced USV production was reduced by yohimbine but not *nor*-BNI. This pattern of results suggests that the α_2-noradrenergic system modulates κ-opioid-mediated USV production, but that the κ-opioid

system does not modulate α_2-noradrenergic-mediated USV production.

It is possible that USVs are emotionally-mediated calls that require the joint activation of both affective and cardiovascular centers in the brain. This conclusion is supported by findings showing that both distress and anxiety increase norepinephrine levels in brain regions involved in affective and cardiovascular functioning. In turn, USV emissions may ultimately be produced by changes in cardiovascular functioning and the onset of ACRs.

Appendix A

ANOVA Table for USV Data of Experiment 1

Source	SS	*df*	MS	F	*p*
Total	16352588.98	39	2831246.44		
Subject	3388146.98	7	484021.00		
Agonist (Agon)	8792978.35	4	2198244.60	14.76	< 0.001
$\text{Error}_{\text{Agon}}$	4171463.65	28	148980.84		

Appendix B

ANOVA Table for Line-Cross Data of Experiment 1

Source	SS	*df*	MS	F	*p*
Total	27553.10	39	3483.16		
Subject	3209.90	7	458.56		
Agonist (Agon)	10057.60	4	2514.40	4.93	< 0.01
$Error_{Agon}$	14285.60	28	510.20		

Appendix C

ANOVA Table for Rectal Temperature Data of Experiment 1

Source	SS	*df*	MS	F	*p*
Total	31.36	39	5.20		
Subject	6.71	7	0.96		
Agonist (Agon)	15.68	4	3.92	12.24	< 0.01
$Error_{Agon}$	8.97	28	0.32		

Appendix D

ANOVA Table for USV Data of Experiment 2

Source	SS	*df*	MS	F	*p*
Total	17462694.01	287	5811937.04		
Subject	266870.72	7	38124.39		
Antagonist (Antag)	2884143.56	2	1442071.80	43.91	< 0.001
Agonist (Agon)	5928019.77	2	2965009.90	55.91	< 0.001
Time	8275.03	3	2758.34	1.39	< 0.275
Antag × Agon	4677555.42	4	1169388.90	20.87	< 0.001
Antag × Time	62832.24	6	10472.04	2.14	< 0.068
Agon × Time	143260.45	6	23876.74	6.20	< 0.001
Antag × Agon × Time	54524.28	12	4543.69	1.48	< 0.146
$Error_{Antag}$	459798.55	14	32842.75		
$Error_{Agon}$	742234.84	14	53016.77		
$Error_{Time}$	41781.81	21	1989.61		
$Error_{Antag \times Agon}$	1569172.64	28	56040.81		
$Error_{Antag \times Time}$	205296.09	42	4888.00		
$Error_{Agon \times Time}$	161788.05	42	3852.10		
$Error_{Antag \times Agon \times Time}$	257140.56	84	3061.20		

Appendix E

ANOVA Table for Line-Cross Data of Experiment 2

Source	SS	*df*	MS	F	*p*
Total	124137.62	71	38423.85		
Subject	1905.78	7	272.25		
Antagonist (Antag)	16727.86	2	8363.93	11.90	< 0.001
Agonist (Agon)	40016.72	2	20008.36	68.44	< 0.001
Antag × Agon	32400.66	4	8100.16	11.84	< 0.001
$Error_{Antag}$	9836.81	14	702.63		
$Error_{Agon}$	4092.95	14	292.35		
$Error_{Antag \times Agon}$	19156.84	28	684.17		

Appendix F

ANOVA Table for Rectal Temperature Data of Experiment 2

Source	SS	*df*	MS	F	*p*
Total	94.84	71	26.42		
Subject	26.16	7	3.74		
Antagonist (Antag)	0.15	2	0.08	0.19	< 0.829
Agonist (Agon)	41.59	2	20.79	30.21	< 0.001
Antag × Agon	1.40	4	0.35	0.95	< 0.450
$Error_{Antag}$	5.57	14	0.40		
$Error_{Agon}$	9.64	14	0.69		
$Error_{Antag \times Agon}$	10.33	28	0.37		

Appendix G

ANOVA Table for USV Data of Experiment 3

Source	SS	*df*	MS	F	*p*
Total	10233072.89	251	3580205.23		
Subject	252476.22	6	42079.37		
Antagonist (Antag)	1921607.72	2	960803.86	40.11	< 0.001
Agonist (Agon)	4505085.77	2	2252542.90	49.71	< 0.001
Time	10350.23	3	3450.08	0.85	< 0.485
Antag × Agon	640540.49	4	160135.12	2.55	< 0.066
Antag × Time	16963.71	6	2827.28	1.17	< 0.346
Agon × Time	49387.28	6	8231.21	3.18	< 0.013
Antag × Agon × Time	79476.46	12	6623.04	2.94	< 0.002
$Error_{Antag}$	287476.11	12	23956.34		
$Error_{Agon}$	543728.56	12	45310.71		
$Error_{Time}$	73045.46	18	4058.08		
$Error_{Antag \times Agon}$	1509995.17	24	62916.47		
$Error_{Antag \times Time}$	87304.68	36	2425.13		
$Error_{Agon \times Time}$	93251.28	36	2590.31		
$Error_{Antag \times Agon \times Time}$	162383.65	72	2255.33		

Appendix H

ANOVA Table for Line-Cross Data of Experiment 3

Source	SS	*df*	MS	F	*p*
Total	331940.42	62	118265.33		
Subject	21131.97	6	3521.99		
Antagonist (Antag)	22781.56	2	11390.78	9.20	< 0.004
Agonist (Agon)	178406.89	2	89203.44	29.21	< 0.001
Antag × Agon	35692.25	4	8923.06	9.55	< 0.001
$Error_{Antag}$	14854.89	12	1237.91		
$Error_{Agon}$	36642.89	12	3053.57		
$Error_{Antag \times Agon}$	22429.97	24	934.58		

Appendix I

ANOVA Table for Rectal Temperature Data of Experiment 3

Source	SS	*df*	MS	F	*p*
Total	67.38	62	13.66		
Subject	19.26	6	3.21		
Antagonist (Antag)	0.28	2	0.14	0.18	< 0.841
Agonist (Agon)	15.71	2	7.85	27.00	< 0.001
Antag × Agon	2.85	4	0.71	1.04	< 0.406
$Error_{Antag}$	9.40	12	0.78		
$Error_{Agon}$	3.49	12	0.29		
$Error_{Antag \times Agon}$	16.39	24	0.68		

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