Importance of the kappa opioid system for ultrasonic vocalizations of young rats: Role of peripherally-versus centrally-located kappa opioid receptors

James Roy Osburn

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IMPORTANCE OF THE KAPPA OPIOID SYSTEM FOR ULTRASONIC VOCALIZATIONS OF YOUNG RATS: ROLE OF PERIPHERALLY-VERSUS CENTRALLY-LOCATED KAPPA OPIOID RECEPTORS

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts
in
Psychology:
Child Development

by
James Roy Osburn
September 2008
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July 15, 2008
ABSTRACT

The theoretical debate about the underlying mechanisms responsible for the production of ultrasonic vocalizations is ongoing. The traditional view is that ultrasonic vocalizations are a component of communication leading to attachment or a survival response, while the other perspective is that ultrasonic vocalizations are a physiological by-product caused mainly by cold stress.

The kappa opioid receptor system is known to mediate ultrasonic vocalization production, because the kappa opioid agonist U50,448 elicits ultrasonic vocalizations. The purpose of this thesis was to determine whether the kappa opioid receptors modulating ultrasonic vocalizations production are located in the central and/or peripheral nervous system. It was hypothesized that peripheral administration of U50,448 or ICI 204,448 (an agonist that does not cross the blood-brain barrier) would increase ultrasonic vocalizations and reduce rectal temperatures of 11-day-old rats. As hypothesized, both ICI 204,448 and U50,448 increased ultrasonic vocalization production, but neither drug affected rectal temperatures. This pattern of results indicates that peripherally-located kappa opioid receptors are involved in the mediation of ultrasonic vocalization production. Interestingly, both ICI 204,448
and U50,488 also increased the locomotor activity of preweanling rats. While this result may suggest that ICI 204,448 crosses the blood-brain barrier, a more parsimonious conclusion is that stimulation of peripherally-located kappa opioid receptors is able to initiate locomotor activity. In conclusion, kappa opioid receptors are located on neuroanatomical structures that could induce ultrasonic vocalizations by either altering the emotional state of the animal or by causing abdominal compression reactions. However, neither U50,448 nor ICI 204,448 decreased rectal temperatures (as the abdominal compression reaction hypothesis would predict), so the present results are more consistent with the traditional view of ultrasonic vocalization production. Specifically, these findings suggest that peripherally-located kappa opioid receptors are, at least partially, responsible for ultrasonic vocalizations by acting on autonomic structures that alter emotional states (e.g., stress). The kappa opioid receptor system is preserved throughout all mammalian species, including humans, thus it is possible that the same neural substrates underlie infant vocalizations across species.
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Attachment is a primary cornerstone of human development. The attachment system appears to provide a primal safety measure hardwired into the brain from birth to satisfy the biological needs involved in survival through proximity to the caregiver (Newman, 2007). Physiological expressions such as crying are often due to distress from discomfort, such as cold or heat exposure, hunger, fear or need for contact with others (comfort or socialization) (Bowlby, 1973, 1988; Lummaa & Vuorialo, 1998; Seifritz, Esposito, Neuhoff, Luthi, Mustovic, Dammann, Bardeleben, Radue, Cirillo, Tedeschi, & Di Salle, 2003). As conceived by Bowlby (1988), attachment theory in infants is the process of proximity-seeking to maintain or provoke a closeness to a known attachment figure (i.e., caregiver), which could be considered a survival mechanism. Through this mechanism, the infant becomes attached to any caregiver that responds to the infant's perceived distress or alarm (cries) with sensitive and responsive social interactions (Bowlby, 1973; Lummaa & Vuorialo, 1998; Seifritz et al., 2003; Stormark, 2007).
Infant crying has long been considered to be a form of communication between parent and infant (Newman, 2007). In the early stages of development, crying is a primitive biological survival response that elicits maternal retrieval to satisfy a discomfort, such as hunger, cold, separation, or other situations that could result in harm or death (Lummaa & Vuorialo, 1998; Newman, 2007; Seifritz et al., 2003). These primitive biological cries develop into maternal bonds and are the building blocks of communication. These cries have unique tonal qualities that elicit different responses from the maternal caregiver (Lummaa & Vuorialo, 1998; Seifritz et al., 2003). Eventually, more sophisticated communications develop (speech and language) and become the tools that humans utilize to share thoughts and ideas (Jurgen & Ploog, 2003). Language is expressed through speech, signaling (gestures), or writing; however, communications can be as primitive as sounds or movements, which are sometimes seen in the animal world. These sounds or movements can be utilized to provoke specific responses such as retrieval or an alert for safety or distress (Jurgen & Ploog, 2003; Newman, 2007).
Brain Circuitry and Human Maternal Behavior

There is little known about what brain circuitry mediates the maternal response to distress calls (cries) of human infants (Lorberbaum, Newman, Horwitz, Dubon, Lydiard, Hamner, Bohning, & George, 2002; Noriuchi, Kikuchi, & Senoo, 2008). It is understood that these cries provoke a maternal response that is related to the survival of the species and helps in the development of attachment (Ainsworth, 1978; Bowlby, 1973; Noirot, 1972; Seifritz et al., 2003; Stormark, 2007). Lorberbaum et al. (2002) utilized magnetic resonance imaging to measure the effects of cries on the brain activity of young first-time human mothers. It was found that cries evoked more emotional symptoms (sadness) than did control noises (Lorberbaum et al., 2002; Seifritz et al., 2003). When control noises were conducted without the cries, they produced more negative emotions (annoyance) than cries (Lorberbaum et al., 2002; Seifritz et al., 2003). Magnetic Resonance Imaging (MRI) showed that crying alone (without the control noise) activated the anterior and posterior cingulate cortex, medial thalamic nuclei, bilateral mesial prefrontal cortex, and right orbitofrontal cortex (Lorberbaum et al., 2002; Noriuchi et al., 2008; Seifritz, et al., 2003).
Human and Animal Homologues

Numerous studies utilize animal models for understanding brain behavior results in humans; however, it can be difficult to prove commonalities between species (Newman, 2007). That being said, vocalizations in animals are a form of communication and it is common for the ultrasonic vocalizations of young animals to be compared to the cries of human infants (Newman, 2007). Although ultrasonic vocalizations of rats are measured in terms of the number and intensity of vocalizations and by maternal retrieval, human infant cries are often measured in terms of maternal responses to questions and by magnetic resonance imaging to determine which areas of the brain are active (Nelson & Panksepp, 1998; Seifritz et al., 2003). Even though the techniques used to study vocalizations differ according to species, the neural mechanisms underlying the vocalizations of humans and other mammals share biological similarities (Newman, 2007). For example, the amygdala is a common mechanism activated during crying in primates, rodents, and humans (Lorberbaum et al., 2002; Newman, 2007; Noriuchi et al., 2008; Seifritz, 2003), as well as the anterior cingulate gyrus, which becomes active in both primates and humans (Newman, 2007). Thus, there is substantial evidence that
crying in various mammalian species shares common neural mechanisms.
CHAPTER TWO
THEORETICAL PERSPECTIVES OF ULTRASONIC VOCALIZATIONS

Although ultrasonic vocalizations of young rodents have been actively studied since 1956 (Rabon, Sawrey, & Webster, 2001), there is a growing theoretical debate about the underlying mechanisms responsible for the production of ultrasonic vocalizations (Blumberg, Sokoloff, & Kent, 1999). The traditional view is that ultrasonic vocalizations are a component of communication or a survival response, such as eliciting maternal retrieval (Lorberbaum et al., 2002; Newman, 2007; Noriuchi et al., 2008). More recently it has been suggested that ultrasonic vocalizations are a physiological effect (i.e., an “acoustic by-product”) caused by a cardiovascular process called the abdominal compression reaction (Blumberg et al., 1999; Blumberg, Sokoloff, & Kent, 2000a).

Traditional View of Ultrasonic Vocalizations

Traditionally, ultrasonic vocalizations have been identified as a system of communication between infant and adult rodents (Hahn & Lavooy, 2005; Hofer & Shair, 1978; Lorberbaum et al., 2002; Newman, 2007; Noriuchi et al.,
2008). For instance, in rodents, such as rats, the anxiety from being under cold stress or separated from the dam or littermates evokes the pup to vocalize and increase their locomotor activity (Allin & Banks, 1971, 1972; Brunelli, Shair, & Hofer, 1994; Hofer & Shair, 1978). Thus, ultrasonic vocalizations were interpreted as a distress response produced by young animals to elicit parental retrieval due to these stressful factors (Allin & Banks, 1971, 1972; Hofer & Shair, 1978; Lorberbaum et al., 2002; Newman, 2007; Noirot, 1972; Noriuchi et al., 2008). Furthermore, these ultrasonic vocalizations appear to have an important survival value for the pups by eliciting maternal care such as directed search (retrieval behavior) and licking. It has also been shown that the playback of ultrasonic vocalizations can induce stimulus-directed search behavior by the dam (Allin & Banks, 1972; Noriuchi et al., 2008; Wohr, Borta, & Schwarting, 2005). Distress calls of rodents also act as a main elicitor of anogenital licking (Bruuette-Lahlou, Vernet-Maury, & Vigouroux, 1992). This licking is vital to the survival of the young pups by inducing defecation.

Considering the negative effect of separation (i.e., survival, separation anxiety, being under cold stress, and lack of other maternal nurturing), it appears that
ultrasonic vocalizations are a valid index of anxiety (Hahn & Lavooy, 2005). In support of the hypothesis that separation-induced ultrasonic vocalizations are a predictor of anxiety, it has been shown that the rate of calling can be attenuated by anxiolytic (anxiety reducing) drugs (Hofer, 1996; Insel, Hill, & Mayor, 1986; Kehne, McCloskey, Baron, Chi, Harrison, Whitten, & Palfreyman, 1991; Olivier, Molewijk, Der Heyden, Van Oorschot, Ronken, Mos, & Miczek, 1998; Vivian, Barros, Manitiu, & Miczek, 1997). More recently, Rabon et al. (2001) showed that when ultrasonic vocalizations were at their highest rate of production, the retrieval response was the shortest, thus providing additional evidence that the function of infant ultrasonic vocalizations is to facilitate parental responsiveness to retrieve the pups.

Although ultrasonic vocalizations are hypothesized to elicit a maternal response and guide the mother to the lost pup, it is uncertain what characteristics of ultrasonic vocalizations elicit maternal behaviors. According to Brudzynski, Kehoe, and Callahan (1999), it is difficult to differentiate the critical components due to the extreme variability in acoustic parameters. It appears that rat pups do not utilize a single acoustic parameter to code the quantitative message dimensions, but all
parameters (single-call duration, sound-peak frequency, and bandwidth) aid the mother in determining the pup's location. By using a recording system (sonograph) that is capable of monitoring a wide range of frequency modulations, Brudzynski et al. (1999) were able to determine those call characteristics produced by the pups that are important for survival by producing a maternal retrieval response. It was shown that those call characteristics the pups produce, like amplitude, frequency modulation, and short period structure, are all related to the amount of maternal care that the pups experienced during early life (see also Wohr et al., 2005).

It has been speculated that the greater the number of ultrasonic vocalizations the shorter the latency until maternal responding. To determine if the greater number of pup vocalizations would alone determine the length of time taken before maternal response, research conducted by Keller, Saucier, Sheerin, and Yager (2004) utilized heat-induced convulsions to determine if increased body temperature would increase ultrasonic vocalization production and enhance mother-pup interactions. Pups subjected to the higher temperatures did elicit more ultrasonic vocalizations than controls that were not
subjected to the higher temperatures. However, it was shown by Keller et al. (2004) that mother-pup interactions did not significantly differ between heat-induced and control groups despite greater numbers of ultrasonic vocalizations induced by these higher temperatures. Interestingly, it appears that female pups, despite calling more frequently than their male littermates, received the lowest amount of maternal care (Keller et al., 2004), possibly due to the less efficient ultrasonic vocalization production or insufficient call characteristics (see Brudzynski et al., 1999). This heat-induced convulsion study shows that heat stress provokes a reflexive form of vocalization, but it appears that the structural acoustic intensity of the ultrasonic vocalizations or the characteristics of the ultrasonic vocalizations determine what behavior will be elicited from the dam and that the number of ultrasonic vocalizations are not the controlling factor (Keller et al., 2004).

When considering the negative effects of separation, the lack of maternal response elicited by heat-induced ultrasonic vocalizations, and the various acoustic parameters utilized (single-call duration, sound-peak frequency, and bandwidth), these various results support
the traditional view that ultrasonic vocalizations are a true form of intraspecies communication and that these structured signals convey a recognizable sign that provokes predictable behaviors in the recipient.

Ultrasonic Vocalizations As A Physiological Side Effect

Blumberg and colleagues have challenged the traditional view that ultrasonic vocalizations are a distress response produced by young animals to elicit parental retrieval (see Allin & Banks, 1971, 1972; Hofer & Shair, 1978; Noirot, 1972). Instead, they have argued that ultrasonic vocalizations are an "acoustic by-product" of a cardiovascular process called the abdominal compression reaction (Blumberg et al., 1999; Blumberg, Sokoloff, & Kent, 2000b). According to this interpretation, cold stress causes a reduction in heart rate and venous blood flow thus reducing body temperature. To combat this situation, the young animal involuntarily engages in abdominal compression reactions that result in increased blood pressure and therefore a concomitant increase in ultrasonic vocalizations (an involuntary reaction due to the body trying to minimize the cold stress) (Blumberg et al., 1999, 2000b).
Further studies conducted by Blumberg et al. (1999) found that brain activity was altered at various temperatures. After prolonged periods of exposure to temperatures of 18-22°C, with core temperatures falling to levels below 27°C, pups vocalize while exhibiting high levels of locomotor activity (Blumberg et al., 1999; Sokoloff, Kirby, & Blumberg, 1998). Karlsson and Blumberg (2003) found increased spontaneous hippocampal activity along the dentate gyrus axis at body temperatures of 37° and 27°C, indicating that cold temperature also induced reciprocal activation of the hippocampal network, causing oscillations between sustained rhythms and sharp brain waves.

The increase in ultrasonic vocalizations not only takes place when the animal is under extreme cold exposure but also after administration of drugs like clonidine (Kehoe & Harris, 1989). Clonidine is a α₂-adrenoceptor agonist that mimics the action of norepinephrine by directly binding to the receptor. According to Blumberg and colleagues, increases in ultrasonic vocalizations are elicited by, and associated with, enhanced venous pressure (Kirby & Blumberg, 1998). Enhanced venous pressure, in turn, results from the body trying to regain equilibrium by involuntarily compensating through a process known as
abdominal compression reactions, which is due to decreased cardiac rate and stroke volume (Blumberg et al., 1999, 2000b). According to Blumberg et al. (2000a) both cold exposure and clonidine are capable of decreasing cardiac rate and stroke volume and, through these mechanisms, cause an increase in ultrasonic vocalization production. Teitel, Sidi, Chin, Brett, Heymann, and Rudolph (1985) found that infant mammals are limited in their ability to increase stroke rate above basal levels, thus inhibiting their ability to compensate for temperature-or clonidine-induced reductions in cardiac output.

Allin and Banks (1971) suggested that thermal cues are the primary factor determining whether ultrasonic vocalizations are emitted and that other factors (e.g., isolation from the nest, vestibular, olfactory, and tactile information) are not as critical for eliciting the production of ultrasonic vocalizations. Blumberg, Efimova, and Alberts (1992a, b) found that isolation from the nest, dam, and littermates must be accompanied by significant cold stress for the resulting ultrasonic vocalizations to be of significant volume and frequency to instigate a maternal response. More recent studies have refined the process of studying how young rodents react to temperature-induced stressors. For instance, Blumberg and
Stolba (1996) removed pups from the nest and allowed them to acclimate to a warm environment before being exposed to cold. Using this procedure, ultrasonic production increased dramatically across the transition from moderate to extreme cold exposure when the pup could no longer compensate for the loss of body heat. These and similar studies have concluded that ultrasonic vocalizations are a by-product of a physiological maneuver referred to as abdominal compression reaction (Youmans, Murphy, Davis, Briggs, & Hoye, 1963), which enhances venous return during extreme thermal challenges (Kirby & Blumberg, 1998).

The latter results are consistent with the position of Blumberg and colleagues, however, other evidence is more consistent with the traditional view about ultrasonic vocalizations. For example, the abdominal compression reaction hypothesis does not take into consideration magnitude differences in the frequency, single-call duration, sound-peak frequency, and bandwidth of ultrasonic vocalizations. Moreover, the thermogenic response to isolation at room temperature is not influenced by devocalization and brown adipose tissue stimulation is not necessary for the ultrasonic vocalization response to cold (Hofer & Shair, 1991). Therefore, when all the evidence is considered together,
it is uncertain which theory about ultrasonic vocalization production is correct.
CHAPTER THREE
NEUROTRANSMITTER SYSTEMS

Neurotransmitters are chemicals that are used to modulate, amplify, and relay signals between a neuron and other cells. Agonists are exogenous substances that bind to specific receptors and trigger responses in the cell. For example, in the present thesis two kappa opioid agonists (ICI 204,488 and U50,488) were used. Both agonists attach to kappa opioid receptors and stimulate them.

The neural mechanisms mediating ultrasonic vocalizations have been studied intensively, and various neurotransmitter systems have been implicated (Kehoe & Harris, 1989). Three of the most prominent neurotransmitter systems involved in ultrasonic vocalizations are the γ-aminobutyric acid (GABA), adrenergic, and kappa opioid systems. GABA receptors are widely dispersed across brain and, when stimulated, ultrasonic vocalizations are reduced (Adachi, Tomonaga, Achibana, Denbow, & Furuse, 2006). Stimulation of α2-adrenoceptors affects ultrasonic vocalization production through centrally acting receptors in the brain that monitor catecholamine levels in blood (Kehoe &
Harris, 1989). The kappa opioid system plays a role in ultrasonic vocalization production because kappa opioid receptor agonists significantly increase ultrasonic vocalization emissions (Blumberg, Sokoloff, Kirby, Knoot, & Lewis, 2002; Carden, Barr, & Hofer, 1991; Kehoe & Harris, 1989; Nazarian, Krall, Osburn, & McDougall, 2001).

**γ-Aminobutyric Acid**

GABA is the major inhibitory neurotransmitter in the central nervous system; it is essential for the overall balance between neuronal excitation and inhibition (Adachi et al., 2006). Drugs that stimulate GABA receptors decrease ultrasonic vocalizations (Carden & Hofer, 1991). For example, ultrasonic vocalizations can be elicited by injecting histamine, glutamate, or acetylcholine agonists; however, all of these chemically-induced vocalizations can be blocked by the GABA agonist muscimol (Adachi et al., 2006; Whiting, 2003). Consistent with these findings, GABA receptor antagonists are capable of attenuating the suppressive effects of flesinoxan (a GABA agonist) on isolation-induced vocalizations (Adachi et al., 2006). These findings suggest that GABAergic mechanisms are involved in mediating ultrasonic vocalization production.
$\alpha_2$-Adrenoceptors

Stimulation of $\alpha_2$-adrenoceptors increases ultrasonic vocalization production in preweanling rat pups; however, it is uncertain whether these vocalizations are due to modulating distress and anxiety (e.g., Kehoe & Harris, 1989), or by altering cardiovascular functioning (e.g., Blumberg & Sokoloff, 2001; Blumberg et al., 2000b). Regardless, it does appear that the critical $\alpha_2$-adrenoceptors mediating ultrasonic vocalization production are located centrally rather than peripherally. Specifically, peripheral administration of the lipophilic $\alpha_2$-adrenoceptor agonist clonidine, which easily crosses the blood-brain barrier, increases ultrasonic vocalizations; whereas, peripheral administration of the hydrophilic $\alpha_2$-adrenoceptor agonist ST-91, which does not readily cross the blood-brain barrier, does not affect ultrasonic vocalizations (Krall, Andicochea, & McDougall, 2005). Both clonidine and ST-91 increase ultrasonic vocalizations when directly administered into the third ventricle, thus confirming that ultrasonic vocalization production is modulated by $\alpha_2$-adrenoceptors located in brain (Krall et al., 2005).

Clonidine is a centrally acting $\alpha_2$-adrenoceptor agonist that selectively stimulates receptors in brain.
that monitor catecholamine levels in blood. In humans, clonidine is used for the treatment of Attention Deficit Hyperactivity Disorder to alleviate opiate withdrawal symptomology (tachycardia) and for hypertension (Coull, Sahakian, Middleton, Young, Park, McShane, Cowen, & Robbins, 1995; Hunt, Minderra, & Cohen, 1985; Ozdogan, Lahdesmaki, & Scheinin, 2003; Wang, Yuan, & Su, 2003; Zalunardo, Zollinger, Spahn, Seifert, & Pasch, 2000). Clonidine also differentially affects the ultrasonic vocalizations of rats depending on age (Hard, Engel, & Lindh, 1988). Results showed that clonidine elevated ultrasonic vocalizations in 2- to 4-day-old pups, whereas 6-day-old pups showed a pronounced increase in clonidine-induced ultrasonic vocalizations that continued to increase with age. Clonidine also enhanced the number of ultrasonic vocalizations in 12-day-old rats, an age when the number of vocalizations normally decreases (Hard et al., 1988). Interestingly, the excitatory effects of clonidine on ultrasonic vocalization production stopped abruptly at 20 days of age (Hard et al., 1988), most likely due to the rat transitioning to later developmental stages when ultrasonic vocalizations do not confer an evolutionary advantage. Consistent with these findings, it was also reported that the \( \alpha_2 \)-adrenoceptor agonist
clonidine increased ultrasonic vocalizations in 10-day-old rat pups. Unlike in the normal isolated rat pup, however, the ultrasonic vocalizations of clonidine-treated pups appeared resilient to maternal cues, because the pups continued to vocalize even following retrieval (Hansen, 1993).

In summary, the α₂-adrenoceptor agonist clonidine substantially increases the ultrasonic vocalization production of young rats (Thiessen & Upchurch, 1981). The neural mechanisms responsible for this clonidine-induced effect are uncertain but a number of possibilities are apparent. First, clonidine may stimulate α₂-adrenoceptors in brain regions responsible for mediating distress and anxiety (Kehoe & Harris, 1989). Second, α₂-adrenoceptor agonists are capable of decreasing heart rate and blood pressure through central mechanisms (McAuley, Macrae, & Reid, 1989; Wang, Macmillan, Fremeau, Magnuson, Lindner, & Limbird, 1996), which may indirectly enhance ultrasonic vocalization production by initiating abdominal compression reactions (see Blumberg et al., 1999, 2000a). Third, stimulation of α₂-adrenoceptors located on postganglionic fibers causes hypotension and bradycardia (Blumberg et al., 2000a), thus potentially increasing ultrasonic vocalizations through peripheral mechanisms.
The latter possibility appears unlikely, however, because ultrasonic vocalization production is not altered after peripheral administration of ST-91 (a $\alpha_2$-adrenoceptor agonist that does not readily cross the blood-brain barrier) (Krall et al., 2005).

Kappa Opioid System

The kappa opioid system is responsible for regulating reward, locomotor activity, and pain perception (Gardner, 2005; Przewlocki, 2004). These receptors are located in the periphery on pain neurons, in the spinal cord, and in the brain (Zhu & Pan, 2004). It has been shown that systemic (peripheral) administration of kappa opioid agonists, such as U50,488, increase the ultrasonic vocalizations of preweanling rats (Barr, Wang, & Carden, 1994; Carden et al., 1991; Carden, Bortot, & Hofer, 1993; Carden, Davachi, & Hofer, 1994; Kehoe & Boylan, 1994; Kehoe & Harris, 1989; Nazarian et al., 2001; Nazarian, Rodarte-Freeman, & McDougall, 1999). However, these studies have been unable to determine whether the critical kappa opioid receptors modulating ultrasonic vocalizations are located in the central nervous system or peripheral nervous system, because (a) kappa agonists are typically administered systemically (peripherally) and (b) U50,488
freely crosses the blood-brain barrier. Clearly, at least one of the populations of kappa opioid receptors are located centrally, because infusing U50,488 into the midbrain periaqueductal gray readily elicits ultrasonic vocalizations in one-week-old rats (Goodwin & Barr, 2005).

These findings suggest that kappa opioid receptors located in the periaqueductal gray area play an important role in mediating the production of ultrasonic vocalizations (Goodwin & Barr, 2005). However, two questions remain. First, are the kappa opioid receptors mediating ultrasonic vocalizations production exclusively located in the central nervous system (brain and spinal cord) or are there peripherally located kappa opioid receptors that also mediate ultrasonic vocalization production? Second, do these various kappa opioid receptors affect ultrasonic vocalization production by modulating distress or cardiovascular functioning (activation of receptors in the brain or peripherally located receptors on the heart)? In the latter case, kappa opioid receptors located on postganglionic fibers in the brain and in heart tissue are known to induce both bradycardia and hypotension (Pugsley, Penz, Walker, & Wong, 1992). Furthermore, there is evidence that stimulation of kappa 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 & Faden, 1982; Wang & Ingenito, 1994), thus providing evidence that centrally located kappa receptors can affect heart rate. Thus, systemic or central treatment with selective kappa opioid agonists may stimulate ultrasonic vocalization production by depressing cardiovascular functioning (Hannsen, Feuerstein, & Faden, 1984). It also remains possible that stimulation of centrally-located kappa opioid receptors induces ultrasonic vocalization production by directly modulating stress or anxiety circuits in the brain.

Interestingly, U50,488 has been implicated in the mediation of locomotor activity as well as ultrasonic vocalizations. It has been shown that administration of U50,488 increases locomotor activity in preweanling rats (Carden et al., 1991; Jackson & Kitchen, 1989; Nazarian et al., 1999), while depressing locomotor activity in adult rats (Di Chiara & Imperato, 1988; Leyton & Stewart, 1992). The locus of U50,488's locomotor activating effect has been of much interest. For example, Barr, Miya, and Paredes (1992) suggest that kappa opioid agonists increase locomotor activity through both spinal and supraspinal mechanisms. Conversely, microinjecting U50,488 into the
substantia nigra pars reticulata increase locomotor activity of both preweanling and adult rats (Collins, Zavala, Nazarian, & McDougall, 2000).
The purpose of this thesis was to determine whether stimulating kappa opioid receptors in the peripheral nervous system is capable of eliciting ultrasonic vocalization production. This thesis was not designed to directly determine whether kappa opioid receptor stimulation induces ultrasonic vocalizations by modulating distress systems or cardiovascular functioning except to the extent that peripherally located receptors would not typically be part of a neural network (central nervous system) responsible for inducing voluntary motor activity, mediating distress or emotion.

To determine whether centrally- or peripherally-located kappa opioid receptors mediate ultrasonic vocalization production, I compared the effects of U50,488 (a kappa opioid agonist that freely crosses the blood-brain barrier) with ICI 204,448 (a kappa opioid agonist that does not readily cross the blood-brain barrier) (Shaw, Carroll, Alcock, & Main, 1989). More precisely, 11-day-old rats were injected with saline (a control substance), U50,488, (a lipophilic compound) or various doses of ICI 204,448 (a lipophobic compound).
Ultrasonic vocalization production was assessed for 20 minutes after which rectal temperatures were measured. It was hypothesized that administration of ICI 204,448 would increase the production of ultrasonic vocalizations by lowering the heart rate and decreasing body temperature. This pattern of results would mean that ultrasonic vocalizations are partially mediated by peripherally-located kappa opioid receptors. This finding would be in opposition to the current belief that centrally-located kappa opioid receptors exclusively mediate ultrasonic vocalization production (see Goodwin & Barr, 2005).

Although ICI 204,448 is proposed to be unable to cross the blood-brain barrier (Shaw et al., 1989), we measured U50,488- and ICI 204,448-induced locomotor activity as a control to assess whether ICI 204,448 was able to enter the central nervous system. Specifically, U50,488 dramatically increases the locomotor activity of preweanling rats (Jackson & Kitchen, 1989; Kehoe & Boylan, 1994; Kehoe & Harris, 1989; Nazarian et al., 2001; Nazarian et al., 1999; Zavala, Yoshida, Osburn, & McDougall, 2002), and microinjection studies indicate that this kappa opioid agonist induces locomotor activity by stimulating receptors in the substantia nigra pars
reticulata (Collins et al., 2000). In terms of ICI 204,448, however, this hydrophobic kappa opioid agonist has limited ability to cross the blood-brain barrier and gain access to the central nervous system (Barber, Bartoszyk, Greiner, Mauler, Seyfrie, Simon, Gottschlich, Harting, & Lues 1994; Shaw et al., 1989); therefore, ICI 204,448 should not induce locomotor activity in preweanling rats if: (a) ICI 204,488 does not cross the blood-brain barrier and (b) the kappa opioid receptor mediating locomotor activity are exclusively located in the central nervous system.
CHAPTER FIVE
GENERAL METHODS

Subjects
Subjects were 40 rat pups of Sprague-Dawley descent, born and raised at California State University, San Bernardino. Litters were culled to 10 rat pups at postnatal day 4 (day 0 = parturition). Only half (5) of the pups from each litter were utilized as subjects. One rat pup from each litter was randomly assigned to each treatment group. There were an 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. Subjects were treated according to the National Institute of Health’s guidelines, “Guide for the Care and Use of Laboratory Animals” (National Research Council, 1996), under a research protocol that was approved by the Institutional Animal Care and Use Committee of California State University, San Bernardino.

Apparatus
Ultrasonic vocalizations were assessed in a clear Plexiglas chamber (19 x 16 x 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. Lines divided the floor of the testing apparatus into four equal quadrants. 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 & Shair, 1978). Line-crosses were visually recorded by observer’s blind to drug treatment conditions. Rectal temperatures were assessed using a BAT-12 microprobe thermometer (Physitemp Instruments, Piscataway, NJ, USA). The ultrasonic vocalization and rectal temperature data were than entered and assessed.

Drugs
ICI 204,448 and U50,488 were purchased from Sigma (St. Louis, MO, USA). All drugs were dissolved in sterile saline and injected intraperitoneally (i.p.) at a volume of 5 ml/kg.

Procedure
The purpose of this experiment was to determine whether ICI 204,448 would increase the ultrasonic vocalizations of preweanling rats. Eight litters of 11-day-old rats were used, with five rats from each litter
(N = 40) being tested. Specifically, one rat from each litter was injected with saline; one rat from each litter was administered U50,488 (2.5 mg/kg); the three remaining rats from each litter were given one of three doses of ICI 204,448 (1.25, 2.5, or 5 mg/kg). Only one dose of U50,488 was used, because it has been established that 2.5 mg/kg U50,488 produces substantial numbers of ultrasonic vocalizations in preweanling rats (Nazarian et al., 1999, 2001). A broader range of ICI 204,448 (1.25-5 mg/kg) was used, because this compound has been used infrequently in behavioral studies (Shaw et al., 1989). After being injected, rats were returned to their home cage for 15 minutes. After 15 minutes, each rat was individually taken to the experimental room and placed in the testing chamber. During each 20-minute session ultrasonic vocalizations and line-crosses (a measure of forward locomotion) were measured with rectal temperatures being recorded immediately after behavioral testing.

After testing, each rat was anesthetized using sodium pentobarbital and returned to the home cage. This procedure maintained litter size while eliminating the ultrasonic vocalizations of the returning rat, so neither declining litter size nor ultrasonic vocalizations would affect the remaining pups. Specifically, it has been shown
that decreasing litter size and increased ultrasonic vocalizations can cause distress in the remaining test subjects in that litter (see Carden et al., 1993; Krall et al., 2005; Nazarian et al., 2001).

In summary, this experiment utilized one between-subject independent variable with five levels (saline, 2.5 mg/kg U50488, 1.25 mg/kg ICI 204,488, 2.5 mg/kg ICI 204,488, 5 mg/kg ICI 204,488). Ultrasonic vocalization and line-cross data from each 20-minute session was divided into four 5-minute time blocks, so time served as a within-subject independent variable with four levels. The dependent variables were ultrasonic vocalizations, line-crosses, and rectal temperatures.

Statistical Analysis
Analyses of variance (ANOVAs) were used for statistical analysis of ultrasonic vocalization, line-cross, and rectal temperature data. In this experiment, ultrasonic vocalizations and line-crosses were analyzed for 20 minutes, with each 20-minute session being divided into 4 separate 5-minute time blocks. Therefore, a two-way ANOVA (drug x 5 minute time block) was used to analyze both ultrasonic vocalizations and line-cross, data. Rectal temperature data were analyzed using a
one-way (drug) ANOVA. For these analyses, litter effects were controlled by using within-litter statistical procedures (i.e., a within analysis using one value/condition/litter), which considers litter, rather than each subject, as a degree of freedom (Zorrilla, 1997). Tukey tests were used for making post hoc comparisons.
Although it was hypothesized that ICI 204,448 would increase ultrasonic vocalizations and decrease rectal temperatures, this was only partially the case. Ultrasonic vocalizations of rats given peripheral injections of ICI 204,448 (1.25, 2.5, or 5 mg/kg) or U50,488 (2.5 mg/kg) did emit more ultrasonic vocalizations than saline-treated rats, drug main effect, $F(4,28) = 15.32, p < .001$ (see Figure 1). The effects of the drug variable did not vary across the testing session. Rectal temperatures of the rats did not vary according to drug treatment (see Table 1).

Interestingly, when compared to saline controls, all doses of ICI 204,488 (1.25, 2.5, or 5 mg/kg) and U50,488 (2.5 mg/kg) increased the line-crosses of preweanling rats, drug main effect, $F(4,28) = 7.75, p < .001$ (see Figure 2). On time block 2, rats given 1.25 mg/kg ICI 204,448 had more line-crosses than rats given 5 mg/kg ICI 204,448, drug x time block interaction, $F(12,84) = 2.78, p < .01$. All of the drug groups differed from saline controls on time blocks 1-4.
Table 1. Mean (± SE) Rectal Temperatures (°C) of Preweanling Rats (n = 8 Per Group) Injected Intraperitoneally (ip) with Saline, ICI 204,448 or U50,488

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rectal Temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>36.20(± 0.16)</td>
</tr>
<tr>
<td>1.25 mg/kg ICI 204,448</td>
<td>36.01(± 0.20)</td>
</tr>
<tr>
<td>2.5 mg/kg ICI 204,448</td>
<td>35.82(± 0.18)</td>
</tr>
<tr>
<td>5.0 mg/kg ICI 204,448</td>
<td>36.39(± 0.13)</td>
</tr>
<tr>
<td>2.5 mg/kg U50,488</td>
<td>36.04(± 0.27)</td>
</tr>
</tbody>
</table>
Figure 1. Mean (+ S.E.M.) Number of Ultrasonic Vocalizations of 11-Day-Old Rats (n = 8 Per Group) Injected with Saline, ICI 204,488 (1.25, 2.5, or 5 mg/kg), or U50,488 (2.5 mg/kg) 15 Minutes Prior to Testing. Ultrasonic Vocalizations were Assessed for 20 Minutes (Divided into Four 5-Minute Time Blocks)
11-Day-Old Rats (n = 8 Per Group) Injected with Saline, ICI 204,488 (1.25, 2.5 & 5 mg/kg), or U50,488 (2.5 mg/kg) 15 Minutes Prior to Testing. Line-Crosses Were Assessed for 20 Minutes (Divided into Four 5-Minute Time Blocks)

Figure 2. Mean (± S.E.M.) Number of Line-Crosses of 11-Day-Old Rats (n = 8 Per Group) Injected with Saline, ICI 204,488 (1.25, 2.5 & 5 mg/kg), or U50,488 (2.5 mg/kg) 15 Minutes Prior to Testing. Line-Crosses Were Assessed for 20 Minutes (Divided into Four 5-Minute Time Blocks)
CHAPTER SEVEN
DISCUSSION

The purpose of this thesis was to determine whether stimulating kappa opioid receptors in the peripheral nervous system is capable of eliciting ultrasonic vocalization production. Specifically, it was hypothesized that U50,488 and ICI 204,448 would increase ultrasonic vocalizations and decrease rectal temperatures, thus indicating that peripherally-located kappa opioid receptors are important for ultrasonic vocalization production. It was also predicted that only U50,488, and not ICI 204,448, would increase the locomotor activity of preweanling rats because kappa opioid-mediated locomotor activity is thought to be centrally mediated (Collins et al., 2000; Herrera-Marschitz, Christensson-Nylander, Sharp, Staines, Reid, Hokfelt, Terenius, & Ungerstedt, 1986; Matsumoto, Brinsfield, Patrick, & Walker, 1988; Thompson & Walker, 1990). Results showed that rats given peripheral injections of ICI 204,448 (1.25, 2.5, or 5 mg/kg) or U50,488 (2.5 mg/kg) did emit more ultrasonic vocalizations than saline-treated rats; however, rectal temperatures of rats did not decrease as a result of drug treatment (see Table 1). Interestingly, when compared to
saline controls, all doses of ICI 204,488 (1.25, 2.5, or 5 mg/kg) and U50,488 (2.5 mg/kg) increased the line-crosses of preweanling rats. These U50,488-induced effects were expected because this drug readily crosses the blood-brain barrier. However, ICI 204,488 does not cross the blood-brain barrier of adult rats (Barber et al., 1994; Shaw et al., 1989), so the increased locomotor activity after ICI 204,488 administration was unanticipated.

As hypothesized, the results suggest that kappa opioid receptors in the peripheral nervous system mediate ultrasonic vocalization production in 11-day-old rats. Specifically, systemic administration of ICI 204,448, a kappa opioid agonist purported to be unable to cross the blood-brain barrier, elicited substantial ultrasonic vocalization production in preweanling rats. This result was expected because systemic administration of U50,488, a kappa opioid agonist that stimulates both peripherally- and centrally-located kappa opioid receptors, also induces ultrasonic vocalizations (Barr et al., 1994; Nazarian et al., 2001). Interestingly, Goodwin and Barr (2005) report that direct infusion of U50,488 into the periaqueductal gray elicits ultrasonic vocalizations in young rats, thus indicating that centrally located kappa opioid receptors also mediate ultrasonic vocalization production.
Therefore, the most parsimonious conclusion is that ultrasonic vocalizations of young rats are mediated through both central and peripheral mechanisms.

The latter conclusion needs to be tempered, however, because of the locomotor activity data. Specifically, ICI 204,448's ability to increase the locomotor activity of preweanling rats indicates that either (a) ICI 204,448 crossed the blood-brain barrier of preweanling rats or (b) peripherally-located kappa opioid receptors are capable of mediating locomotor activity. Although ICI 204,488 is unable to cross the blood-brain barrier of adult rats (Barber et al., 1994; Shaw et al., 1989), no studies have assessed ICI 204,448's ability to penetrate the blood-brain barrier of preweanling rats. In general, the blood-brain barrier matures rapidly during embryogenesis (Risau & Wolburg, 1999), but it has been reported that ontogenetic changes in blood-brain barrier permeability can occur across the preweanling period (Johanson, 1980; Stewart & Hayakawa, 1987). An alternative possibility is that stimulating kappa opioid receptors in the periphery is capable of inducing locomotion. In this regard, it is important to note that stimulation of peripherally-located kappa opioid receptors induces an aversive state (Bechara & van der Kooy, 1987). Although
speculative, it is possible that this aversive state indirectly results in locomotion. When combined with data showing that U50,488-induced locomotor activity was only partially attenuated by lesions of the ventromedial thalamus or superior colliculus (Zavala et al., 2002), it remains plausible that different populations of peripherally-located kappa opioid receptors separately mediate locomotion and ultrasonic vocalization production.

When the various results are considered together, it appears that kappa opioid receptors in the peripheral nervous system are capable of mediating ultrasonic vocalization production. The question remains as to whether this ultrasonic vocalization production is due to the activation of stress/emotion circuitry or the onset of abdominal compression reactions. As mentioned in the Introduction, Blumberg and colleagues (1999, 2000a, 2000b) have proposed that ultrasonic vocalizations are an "acoustic by-product" of a cardiovascular process called the abdominal compression reaction. Abdominal compression reactions are caused by any factor (i.e., cold stress, drug action, etc.) that reduces heart rate and venous blood flow. These abdominal compression reactions, in turn, increase blood pressure and cause a concomitant
increase in ultrasonic vocalizations (Blumberg et al., 1999, 2000b).

In terms of the present thesis, Blumberg’s abdominal compression reaction hypothesis was tenable because kappa opioid receptors are located on heart tissue and postganglionic fibers in the peripheral nervous system and stimulating these receptors induces both bradycardia and hypotension (Pugsley et al., 1992). In the central nervous system, stimulation of kappa 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 & Faden, 1982; Wang & Ingenito, 1994). Therefore, there are neural mechanisms in place that allow Blumberg’s abdominal compression reaction hypothesis to remain tenable (i.e., kappa opioid receptors are located on neuroanatomical structures that could induce abdominal compression reactions and ultrasonic vocalizations). That being said, it is important to realize that neither U50,488 nor ICI 204,488 decreased the rectal temperatures of rats (see Table 1). This finding is not consistent with the abdominal compression reaction hypothesis, because neither kappa opioid agonist appeared to decrease cardiovascular
functioning (i.e., induced abdominal compression reactions).

An alternative explanation is that ultrasonic vocalizations are an emotionally-based distress response (Allin & Banks, 1971, 1972; Hofer & Shair, 1978; Lorberbaum et al., 2002; Newman, 2007; Noirot, 1972; Noriuchi et al., 2008). In terms of peripherally-located kappa opioid receptors, it is possible that stimulating these receptors induces an aversive state (Bechara & van der Kooy, 1987) leading to an emotional response that initiates ultrasonic vocalization production. As reported by Goodwin and Barr (2005), kappa opioid receptors in the periaqueductal gray are also involved in mediating ultrasonic vocalization production. This brain region is important for defense responses, so it is possible that peripheral and central kappa opioid receptors interact to influence ultrasonic vocalization production by modulating neural mechanisms underlying emotion.

Although the kappa opioid receptor system is clearly involved in modulating ultrasonic vocalization production, other neurotransmitter systems play important roles. For example, there is substantial evidence that stimulation of α2-adrenoceptors increases ultrasonic vocalization production in preweanling rat pups, although it is still
uncertain whether these vocalizations are due to modulating distress and anxiety (e.g., Kehoe & Harris, 1989) or by altering cardiovascular functioning (e.g., Blumberg & Sokoloff, 2001; Blumberg et al., 2000b). Evidence for both positions exist, because \( \alpha_2 \)-adrenoceptors are located in brain regions responsible for mediating distress and anxiety (Kehoe & Harris, 1989), while \( \alpha_2 \)-adrenoceptor agonists (e.g., clonidine) are capable of decreasing heart rate and blood pressure (McAuley et al., 1989; Wang et al., 1996). Regardless of explanation, the \( \alpha_2 \)-adrenoceptors mediating ultrasonic vocalization production appear to be exclusively located in the central nervous system. Specifically, peripheral administration of the lipophilic \( \alpha_2 \)-adrenoceptor agonist clonidine, which easily crosses the blood-brain barrier, increases ultrasonic vocalizations; whereas, peripheral administration of the hydrophilic \( \alpha_2 \)-adrenoceptor agonist ST-91, which does not readily cross the blood-brain barrier, does not affect ultrasonic vocalization production (Krall et al., 2005).

Interestingly, there is evidence that the kappa opioid and noradrenergic systems interact to mediate ultrasonic vocalization production. Specifically, Nazarian et al. (2001) reports that an \( \alpha_2 \)-adrenoceptor antagonist
attenuated U50,488-induced ultrasonic vocalizations; whereas, a kappa opioid antagonist did not block clonidine-induced ultrasonic vocalizations. These results suggest that the $\alpha_2$-adrenoceptor system must be functional for the kappa opioid system to modulate ultrasonic vocalizations (Nazarian et al., 2001). In terms of the present study, these results imply that peripherally- and centrally-located kappa opioid receptors interact complexly with other neurotransmitter systems to mediate ultrasonic vocalization production.

As mentioned in the Introduction, ultrasonic vocalizations are an important signal that elicits maternal behavior in adult rats (Lorberbaum et al., 2002; Newman, 2007; Noriuchi et al., 2008). For instance, maternal responses (searching) have been elicited by playing back recordings of ultrasonic vocalizations (Allin & Banks, 1972; Noriuchi et al., 2008; Wohr et al., 2005). Interestingly, maternal responses are based on the individual acoustic parameters of the calls rather than the number of calls (Keller et al., 2004). The ability to differentiate acoustic parameters probably explains why some calls of young rodents elicit maternal retrieval and others act as a main elicitor of anogenital licking, which
is vital to the survival of the young pups (Bruuette-Lahlou et al., 1992).

Analogous processes appear to occur in humans, because the cries of infants can induce profound emotional responses in human mothers (Lorberbaum et al., 2002; Seifritz et al., 2003). In these studies such cries lead to protective responses, whereas the same mothers showed irritable reactions to control noises. Although it is true that crying is often due to distress or the need for socialization (Bowlby, 1973, 1988), cries are also considered a survival mechanism that initiate bonding and survival of the young human (Bowlby, 1973, 1988; Lummaa & Vuorialo, 1998; Seifritz et al., 2003). For instance, Bowlby (1973, 1988) conceived attachment as a process of proximity-seeking, which causes a known caregiver to respond and maintain closeness. In both humans and animals, therefore, vocalizations initiate a complex social interactive process that satisfies the biological needs of the infant (Lummaa & Vuorialo, 1998; Seifritz et al., 2003) by eliciting a maternal response.

In conclusion, there is little doubt that vocalizations (e.g., crying) elicit maternal care in both humans and animals, so it would not be surprising if the mechanisms responsible for vocalization production share a
common neural substrate. In this regards, it is important to remember that the same neural network (including the kappa opioid, α₂-adrenoceptor and GABA systems) are preserved throughout invertebrate and vertebrate species (Ruuskanen, Laurila, Xhaard, Rantanen, Vuoriluoto, Wurster, Marjamaki, Vainio, Johnson, & Scheinin, 2005; Stevens, Brasel, & Mohan, 2007; Tsang, Ng, Xu, & Xue, 2007), and that these neural mechanisms are activated during crying in rodents, nonhuman primates, and humans (Lorberbaum et al., 2002; Newman, 2007; Noriuchi et al., 2008; Seifritz et al., 2003).

In the case of humans, crying has been studied using magnetic resonance imaging, but this technology is not capable of measuring the functioning of individual neurotransmitter systems. For this reason the neural mechanisms underlying human crying are poorly understood; however, because of the preservation of neural networks across species, the present thesis suggests that the kappa opioid neurotransmitter system may be an important neural mechanism mediating crying in humans. Additionally, my results are consistent with the traditional view that the ultrasonic vocalization production of young rats is caused by an emotional response (rather than abdominal compression reactions). Obviously, this conclusion is also
consistent with the human literature, because human crying is believed to be an emotional form of communication (Newman, 2007) signaling distress or alarm (Bowlby, 1973; Lummaa & Vuoritalo, 1998; Seifritz et al., 2003; Stormark, 2007).
REFERENCES


