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Pharmacology of Ultrasonic Vocalizations in adult Rats: Significance, Call Classification and Neural Substrate

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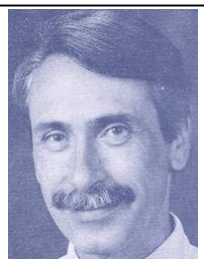

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Abstract: Pharmacological studies of emotional arousal and initiation of emotional states in rats measured by their ultrasonic vocalizations are reviewed. It is postulated that emission of vocalizations is an inseparable feature of emotional states and it evolved from mother-infant interaction. Positive emotional states are associated with emission of 50 kHz vocalizations that could be induced by rewarding situations and dopaminergic activation of the nucleus accumbens and are mediated by D1, D2, and partially D3 dopamine receptors. Three biologically significant subtypes of 50 kHz vocalizations have been identified, all expressing positive emotional states: (1) flat calls without frequency modulation that serve as contact calls during social interactions; (2) frequency-modulated calls without trills that signal rewarding and significantly motivated situation; and (3) frequency-modulated calls with trills or trills themselves that are emitted in highly emotional situations associated with intensive affective state. Negative emotional states are associated with emission of 22 kHz vocalizations that could be induced by aversive situations, muscarinic cholinergic activation of limbic areas of medial diencephalon and forebrain, and are mediated by M2 muscarinic receptors. Two biologically significant subtypes of 22 kHz vocalizations have been identified, both expressing negative emotional sates: (1) long calls that serve as alarm calls and signal external danger; and (2) short calls that express a state of discomfort without external danger. The positive and negative states with emission of vocalizations are initiated by two ascending reticular activating subsystems: the mesolimbic dopaminergic subsystem as a specific positive arousal system, and the mesolimbic cholinergic subsystem as a specific negative arousal system.

Keywords: Appetitive state, aversive state, cholinergic system, dopaminergic system, emotional arousal, 22 kHz calls, 50 kHz calls, ultrasonic calls.

PHARMACOLOGICAL STUDIES OF ORGANISMAL STATE

Broadly understood behavioral pharmacology has a long history of studies aimed at understanding effects of pharmacological agents on animal and human behavior. These studies were particularly focused on general effects of systemic psychoactive drugs, but also on animal models of particular behaviors (e.g., eating behavior, aggressive behavior), and models for studying therapeutic potential of pharmacological agents. Behavioral pharmacology investigations were greatly enriched by introduction of methods of intracerebroventricular and intracerebral drug application that allowed bypassing the blood-brain barrier. It appeared that many different neuroactive agents may induce the same behavior from the same brain site, and also the same agents may cause different behavioral effects when injected into different brain sites or regions [1]. Within the last 50 years, extensive literature has cumulated, reaching probably over a quarter of a million publications that were dealing with complex brain mechanisms and regulations of behavioral patterns and components of normal and abnormal behaviors.

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One of the important directions of behavioral studies is research focused on the central regulation of the general state of the organism. Pharmacological agents were used as principal tools of choice in these studies. This includes drug effects on arousal, sleep and wakefulness, emotional arousal, attention, vigilance, and many other related states of the organism, e.g., [2-8] and others. These studies contributed not only to better understanding functions of brain systems responsible for changes of the organismal state but also contributed to development of pharmacological agents modulating behavior and cognition and promoting treatment of abnormalities of attention, sleep, or emotional states.

Studies of brain systems involved in the control of organismal state have been encountering, however, a serious problem: how to quantitatively and reliably measure the state of the organism, particularly the emotional state? (For definition of the organismal state, see [9]). The present review is focused on emotional arousal and initiation of emotional states in the rat species and offers one of the solutions to this problem by monitoring and studying ultrasonic vocalization emitted by rats, as a sensitive index (or indicator variable [9]) of animal emotional state.

PHARMACOLOGICAL STUDIES OF EMOTIONAL STATES

Studies of animal emotional arousal with concomitant emission of vocalization have also a long history. First

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experiments were performed on cats more than half a century ago, and showed that systemic or central application of different drugs induced emotional-like behavior with emission of species-specific vocalizations, but with a call type characteristic for the drug used. For example, application of the hallucinogenic lysergic acid diethylamide (LSD) induced a peculiar state in cats, akin to rage with bursts of only hissing type of vocalization to the slightest environmental sound [10, 11]. D-tubocurarine injected into cat's hypothalamus elicited motorically active behavioral state with solely mewing-type of vocalization [12, 13], while intracerebral carbachol and a number of cholinergic agents (acetylcholine, eserine) induced defensive-type of response in cats with an almost exclusively growling type of vocalization [14-16]. Since cat's emotional behavior, and particularly aversive one, is well understood by comparison to natural situations, for example, when cat is facing an aggressive dog or during inter-male aggressive encounters [17], the pharmacological data strongly suggested that many drugs directly interact with the elements of the emotive systems of the brain. The drug-specific types of vocalizations have further suggested that particular types of calls induced by particular drugs may have behavioral significance associated with distinct emotional states.

Further extensive studies on rats have confirmed that rat sonic or ultrasonic vocalizations, similarly to cat's vocalizations, may be induced by a number of pharmacological agents also in a drug specific manner. Cannabis induced species-specific warning sonic vocalizations triggered by light touch [18, 19], while systemic or intracerebral application of drugs related to reward, as amphetamine, cocaine, or methylphenidate, induced solely appetitive 50 kHz ultrasonic vocalizations [20-22]. On the other hand, intracerebral injection of carbachol in rats, a predominantly muscarinic agent, induced pure 22 kHz vocalizations [23, 24], an aversive and alarming type of calls [25]. Behavioral effects caused by application of these neuroactive agents included not only drug-specific vocalizations but also a drug-specific emotional state.

An initial convincing evidence of vocal expression of a carbachol-induced specific emotional state akin to anxiety came again from studies on cats. Intracerebral carbachol induced growling vocalization and this vocalization is well-known as a threatening, aversive call [17]. In further studies, the number and time of growling vocalizations emitted by cat injected with carbachol into the anterior hypothalamic-preoptic area was directly proportional to the distance from the researcher (just standing with an extended hand toward the cat) to the observation chamber with the cat [26]. The closer was the researcher to the animal the higher was the number and mostly duration of growling vocalizations. Also, other threatening stimuli as loud sound or appearance of a barking dog were significantly increasing the growling response [27]. Thus, growling type of vocalization was qualitatively and quantitatively expressing a specific emotional state, so that the number and duration of growling vocalizations was measuring the cat's aversive state [16, 27].

BEHAVIORAL AND PHARMACOLOGICAL STUDIES OF POSITIVE EMOTIONAL STATES IN RATS

Studies performed on rats have shown that 50 kHz vocalizations appeared in a number of well-documented appetitive, positive behavioral situations and they were increased by rewarding, hedonic stimuli and supressed by aversive stimuli [28, 29]. The emission of 50 kHz vocalizations was induced or elevated in such situations as juvenile rough-and-tumble play, rat play with human hand ("tickling"), approach and mating, positive (non-combative) social encounters, and in response to replay of 50 kHz calls that appeared having rewarding properties themselves [30-35]. Rats also showed a relative increase, or initial increase in emission of 50 kHz calls during sucrose self-administration or selection of sweet foods [36, 37]. These appetitive calls were also increased by rewarding electrical brain stimulation or in anticipation of such stimulation, application of cocaine or amphetamine, as well as anticipation of alcohol self-administration [35, 36, 38-41]. In alcohol-dependent rats, number of emitted 50 kHz calls positively correlated with the amount of drunken alcohol [41]. Emission of calls was associated with the release of dopamine in the nucleus accumbens as was demonstrated for response to tickling or replay of 50 kHz vocalizations but not 22 kHz calls [42, 43]. Although different rat strains differ in the amount of emitted calls [44], direct injections of amphetamine into the nucleus accumbens reliably induced high numbers of 50 kHz vocalizations [20, 45] (see subsection on pharmacological studies of 50 kHz calls).

Emission of 50 kHz vocalizations is signalling to conspecifics a hedonic, positive emotional state labeled in human terms as joy, pleasure, or euphoria. These calls were also suggested to be an evolutionary homolog of human laughter [30, 46]. Hence, 50 kHz vocalizations caused behavioral activation in the recipients, in their approach to the source of 50 kHz vocalizations, as well as, the rat-recipients emitted increased numbers of these vocalizations in response to the replay of vocalization, and self-administered the replay of 50 kHz calls [32, 34, 35, 47]. Social emission of 50 kHz vocalizations was interpreted as pro-social behavior, pro-contact calls (i.e., calls soliciting social contacts), signals establishing social proximity, or as emission of play signals [47-50].

Finally, results of studies on rat lines selected for many generations for low or high levels of 50 kHz vocalizations (as tested in heterospecific juvenile play – tickling) brought further results. Rat phenotype, that was obtained by such a selection and that had natural tendency to emit high numbers of 50 kHz vocalizations represented animals that made more social contacts with conspecifics and were more active than animals emitting low numbers of 50 kHz, and thus, were interpreted as stress resilient phenotype and possibly resistant to autism spectrum disorders [51, 52].

The original tickling method [30] denotes a play between human hand and juvenile rats that followed the sequence of events of natural rough-and-tumble play of young rats. Although, the method was designed for juveniles only, some experiments have shown that a mild tactile stimulation with human hand of adult rats ("new tickling procedure") caused a limited elevation of emission of 50 kHz vocalizations demonstrating that some adults accepted this as a play [53]. In a recent study, "tickling" was redefined again and this term labeled a "playful handling" of adult rats that also

increased number of emitted 50 kHz calls and showed a buffering, reducing effect on stress induced by repeatable intraperitoneal injections of saline [54].

BEHAVIORAL AND PHARMACOLOGICAL STUDIES OF NEGATIVE EMOTIONAL STATES IN RATS

Contrary to the results of behavioral studies of the 50 kHz calls, emission of rat 22 kHz vocalizations by adult rats unequivocally suggested association of these calls with a negative, aversive state. The 22 kHz vocalizations were recorded from rats facing predators (e.g. a live cat from a safe distance, but not a toy cat), and could be attenuated by systemic morphine [55-57]. It is interesting that, in response to a cat, morphine (5 mg/kg) shortened total duration of calling time, reduced number of calls, and decreased the mean sound frequency of calls but prolonged the duration of individual vocalizations [57]. Morphine could be decreasing the emotional/affective component of the defensive response. Emission of long 22 kHz calls was also reported for any dangerous or threatening stimulus as foot shock [58-60], loud acoustic startling stimuli [61-63], unexpected air-puff [64, 65], mild electric stimulation of the rat tail [66], encounter with a dominant rat [67] or close approach of unfamiliar humans to naïve rats [59, 68]. Emission of 22 kHz vocalizations was also reported for prolonged isolation or chronic pain and these last calls could be attenuated by aspirin or morphine [69, 70].

The aversive 22 kHz vocalizations will appear in any stressful and aversive behavioral situation. Rats defeated by an aggressive opponent, showing submission, or treated with other painful stimuli consistently emitted 22 kHz calls, although these calls were not always easily interpreted in complex situations of combative inter-male encounters [71-73]. Also, repeated postejaculatory 22 kHz calls emitted by males at the end of a copulatory cycle were interpreted as signals of desist contact, absolute refractory period, social depression and a withdrawn state, so calls signalling a negative state [74-76].

Significant numbers of 22 kHz vocalizations were observed during withdrawal from prolonged exposure to addictive agents, such as psychomotor stimulants (e.g., cocaine), alcohol, benzodiazepines, or opiates, particularly in response to startle [77-79]. Also, decreased or insufficient doses of cocaine in addicted rats, triggered emission of 22 kHz calls, as well as, these calls were appearing as a signal of decreasing probability of reinforcement in Pavlovian conditioning [80, 81]. In agreement with these observations, application of agents with aversive effects (naloxone or lithium chloride) increased emission of 22 kHz calls and decreased emission of 50 kHz calls [82].

Further studies have shown that events associated with emission of 22 kHz vocalizations were retained in the animal memory for a longer time (more stable memories) than those associated with other calls, and were resistant to extinction [83]. Production of 22 kHz calls in repeated dangerous situations might reinforce these memories by learning process or "autoconditioning" [84, 85]. These results indicate that emission of 22 kHz vocalizations is associated with negative/aversive events of any nature and they are well remembered by the animals as biologically important events.

Emission of 22 kHz vocalizations is signalling to conspecifics a negative emotional state labeled in human terms as displeasure, anxiety, chronic fear, or dysphoria. Hence, reply of 22 kHz vocalizations to naïve rats caused decrease in their locomotor activity, increase in behavioral inhibition and freezing responses, avoidance of playback of 22 kHz calls, and lack of call self-administration [32, 86]. Also, emission of 22 kHz calls was potentiated by previous stress at the juvenile stage [87]. Social emission of 22 kHz vocalizations was interpreted as alarm calls, expression of affective distress, anticipation of punishment, or social transmission of fear [55, 79, 84, 88, 89]. The 22 kHz type of vocalization can be pharmacologically induced by direct cholinergic stimulation of the brain and the resulting vocalizations are indistinguishable from calls induced in natural situations [59] (see subsection on pharmacological studies of 22 kHz calls).

Finally, results of studies on rat lines selected for many generations for low levels of 50 kHz vocalizations (as tested in heterospecific juvenile play – tickling) brought further results. Rat phenotype that emitted low numbers of 50 kHz vocalizations and higher incidence of 22 kHz calls represented animals that avoided social contacts with conspecifics and were less active than animals emitting high numbers of 50 kHz, and thus, were interpreted as stress prone phenotype and susceptible to autism spectrum disorders, and that condition could be reversed by treatment with NMDA receptor functional glycine site partial agonist, GLYX-13, [51, 52, 90]. In a different study, low 50 kHz vocalizing male rats emitted more 22 kHz vocalizations than high vocalizers in response to a prolonged stress, showed stable reduction of body weight gain, lower sucrose intake, and higher immobility in a swim test that is used to evaluate depressive-like symptoms [91].

SIGNIFICANCE OF RAT VOCAL RESPONSES AS INDICES OF EMOTIONAL STATES

There is at least 50 species of rodents that were reported to emit ultrasonic vocalizations [92], although, likely all rodents are capable of ultrasonic communication. Only rat vocalizations, however, have been demonstrated to be reliable indices of animal emotional states. Results of pharmacological-behavioral studies on rats and other species have clearly demonstrated that emissions of vocalizations were accompanied by emotional states with dramatic changes in the organism, including somatic and autonomic symptoms [9, 93]. Induced vocalizations were not only state-specific but were also a constant concomitant of the response, hence they could serve as valuable behavioral indices. Thus, the question arises, why animals are vocally signalling their emotional states to conspecifics?

The answer to this question comes partially from extensive anatomical and physiological comparative studies and evolutionary trends in all vertebrates. Production of vocal signals is one of the oldest ways of social communication in all tetrapods [94, 95]. Embryological evidence suggests that highly conserved neuronal groups located in the caudal

hindbrain (from embryonic rhombomere 8 to rostral spinal cord) control vocalization in all vocal vertebrates (from bony fishes to humans) and represent common ancestral structures for vocal communication [96, 97]. Neuronal mechanisms of this control evolved very early in vertebrate history, probably at the time of evolutionary appearance of the myelinated ventral vagal complex of the autonomic system that controls gestural head and facial movements, mastication, and laryngeal and pharyngeal muscles instrumental for vocalization [98]. Functions of this ventral vagal system pertain to the control of sensory input from the environment and social engagement, thus this system has been termed social engagement system [98, 99]. It may be postulated that the most advanced function of this system is intraspecific vocal communication. Groups of neurons in the caudal hindbrain have been shown to regulate separately such features as duration of calls and frequency of emitted sounds as early as in vocal fish (e.g., midshipmen fish belonging to a group of toadfish) [100, 101].

It has been further hypothesized that vocal communication evolved as a very early trait of social behavior related to reproduction because of a need for maternal/parental care of infants. This care was of particular evolutionary importance and it was associated with nursing and interacting with newborns. Nursing, audiovisual communication with mother, and play were regarded as three critical developments leading to evolution of early mammals [102]. The social engagement system was critical for this development. Infant distress calls or "cries" are regarded as universal feature among mammalian species and as a vehicle of vocal communication with mothers [103].

Interaction of mothers with infants and further maintenance of social bonds within the group were the prime reasons for gradual development of elaborate vocalization system [104]. There were also other contributing factors to the evolution of vocalization such as size and the complexity of the social group, predator pressure and habitat, etc. (for details of other contributing factors to evolution of rat vocalization see [104]). This evolutionary trend allowed females to give birth to relatively undeveloped neonates (especially altricial infants, as in rats or humans). Mothers that were particularly carefully attending their infants and trying to control them, particularly by vocal signals, were selected for, and by the same token, pups that could not efficiently vocalize back to mother were eliminated. Mother-infant vocal interaction remained after weaning. It has been further hypothesized that this type of selection also led to transition from prelinguistic vocalizations to human protolanguage [105]. Thus, the pressure for vocal communication remained in mammalian evolution and with further developments of the limbic system and emotional states, expression of these states inseparably included vocal signals addressed to members of the social group [95]. Emission of these vocalizations during emotional states is biologically important and has adaptive value. Accordingly, prolonged post-weaning isolation from mother and social group markedly decreased calling [106]. From these reasons, rat ultrasonic vocalizations that are directed to conspecifics may be used as reliable indices of their emotional states not only in adulthood but also at the infantile stage of early ontogenetic development of emotional system and emotional communication [107, 108].

Adaptive value of signaling aversive states (rat 22 kHz vocalizations) is more obvious because it have protective value both in dyadic interactions (e.g., as appeasement call) and for the whole social group as alarm call, a higher order defensive behavior [24]. On the other hand, adaptive value of signaling appetitive state (rat 50 kHz vocalizations) is less documented. Adaptive value of the 50 kHz calls have been shown in some behavioral situations (e.g., mating or juvenile play) but there is no convincing evidence in other situations (e.g., feeding, when rats could signal palatable food to other members of the group). There is, however, evidence that rats use 50 kHz calls for positive cooperative actions, e.g., in order to obtain reward in joined and simultaneous actions [109].

PHARMACOLOGICAL STUDIES OF 50 kHz VOCALIZATIONS AND THEIR CLASSIFICATION

The conserved evolutionary association of vocal signals with emotional states, and experimental results showing drug specificity for some species-typical vocalizations, opened a new avenue for pharmacological-behavioral studies of basic animal emotional states and behaviors with vocalization as an indicator variable [9]. The largest attention of researches is focused on those pharmacological agents that can evoke positive, appetitive states signalled in rats by 50 kHz vocalizations. This line of research is relevant to interest in mechanisms of motivation, reward, hedonic processes, and addiction.

Systemic or intracerebral injection of dopamine agonists, mostly into the shell of the nucleus accumbens, induced emission of abundant numbers of 50 kHz calls as compared to vehicle injection [9, 20-22, 28, 39, 45, 80, 110, 111]. Animals selected for high numbers of 50 kHz vocalizations emitted during heterospecific play appeared particularly sensitive to dopamine agonists and emitted the highest numbers of these calls [112]. The vocal response was dose-dependent and antagonized by dopamine antagonists such as haloperidol, raclopride, or SKF-83566 [45, 113]. There is only one report of induction of 50 kHz calls from the nucleus accumbens by injection of carbachol (a cholinergic agent) at doses 250-1000 times smaller (1-4 ng) than those needed for elicitation of 22 kHz calls (signaling the opposite state) from other regions of the brain [114]. These results, however, could not be reproduced [112] and gave responses similar to those after vehicle injections.

A number of D1, D2 and D3 receptor antagonists could, at least partially and dose-dependently antagonize the response suggesting that these dopamine receptor subtypes are implicated in the generation of pharmacologically-induced 50 kHz vocalizations [45, 110, 115, 116]. Interestingly, however, D1 and D2 selective dopamine receptor agonists given systemically alone or in combinations, were not able to induce 50 kHz vocalizations, while the D4 dopamine receptors were shown not be involved in production of 50 kHz calls [115]. On the other hand, agents with combined action on D1-/D2-like families of receptors, as apomorphine, could induce 50 kHz calls [111]. This may be explained by

observation that none of the dopaminergic agonists has complete efficacy, as compared to dopamine, at all D2-like receptor sites [117], so each of the agonists may slightly differ in its pharmacological effects. In agreement with these results that D1 and D2 receptors are involved in the response, tickling-induced 50 kHz vocalizations were also antagonized by D1 and D2 antagonists [42]. The role of D1 and D2 dopamine receptors in production of 50 kHz vocalizations is in a general agreement with results of earlier studies showing that D1 and D2 receptors antagonists decreased or blocked self-administration of amphetamine into the shell of the nucleus accumbens [118].

A recent study has also indicated that both D1 and D2 receptors are functionally linked in this regulation. Emission of 50 kHz vocalizations by males (induced by presence of an estrous female) was decreased, duration of calls and number of complex calls decreased, and latency to call increased by systemic pretreatment of rats with combination of D1 (SCH-23390) and D2 (eticlopride) dopamine receptor antagonists [119]. Each of the dopamine antagonists alone had lesser or limited effect. This finding is relevant to earlier reports indicating that D1 and D2 dopamine receptors can form hetero-oligomers and work in tandems in a synergistic manner, including the ventral striatum [120, 121]. The D1-D2 receptor oligomers have distinct signaling properties and were also found in the nucleus accumbens and ventral tegmental area [122, 123].

Detailed sonographic studies of 50 kHz calls revealed that these vocalizations demonstrate some acoustic heterogeneity and could be further divided into subtypes [28, 32, 110, 124-127]. The division into subtypes was mostly based on such features as frequency profile and call duration. The 50 kHz vocalizations were divided into flat calls (FL, constant sound frequency) and frequency-modulated calls (FM, variable sound frequency; see Fig. **1**). Both FL and FM 50 kHz vocalizations remain within the same peak frequency range but differ in the sonographic profile and duration. FL 50 kHz calls have duration of approximately 10-100 ms and their frequency range is between 35 and 50 kHz, while the FM 50 kHz calls have approximate duration 20-150 ms and their frequency range is 40-80 kHz with dramatic changes in sound frequency (Fig. **1**) [32, 104, 110, 124, 126, 128].

Both subtypes of 50 kHz vocalizations are associated with positive, appetitive situations, however, there is a difference in the contexts they are emitted. While the FM 50 kHz calls are emitted in strongly rewarding and highly motivated situations (e.g., sexual situations or aggression) [28, 32, 125], the FL 50 kHz calls were reported having social-coordinating role and served as contact calls [124], as well as, their increased emission was observed during feeding behavior and in anticipation of palatable food [128, 129]. In addition to these behavioral differences, recent studies reported pharmacological differences between FL and FM 50 kHz vocalization. Systemic application of propranolol dose-dependently increased FL 50 kHz vocalizations in amphetamine treated rats but suppressed or eliminated FM 50 kHz calls, reversing the amphetamine-induced proportion of FL to FM 50 kHz vocalizations [130]. This result may

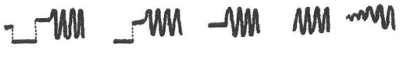
Call type	Pictogram of the call	Selected references
22 kHz short		[68,104,143]
22 kHz long		[65,68,104,143,148]
50 kHz flat		[39,104,119,143,151]
50 kHz FM step		[32,104,119,126,129,130, 143,151]
50 kHz FM with trills		[32,39,104,119,125,126, 129,130,143,151]

Fig. (1). The Figure illustrates diagrammatically main acoustic features of subtypes of ultrasonic vocalizations of adult rats in a tabular way. Each subtype (call type listed in the left rubric) is shown in a form of the most typical pictograms with main sonographic features characteristic for the subtype in the center rubric. Not all possible variations of the calls are depicted. Pictograms are not actual sonograms. Thicker lines denote sound frequency over time. The time bar = 100 ms. The dotted vertical lines join elements of a single call but are not present in actual sonograms. Sonograms recorded from rats show higher variability than that illustrated here and each element of a call may be represented by less straight lines and with a varying degree of turbulence. Elements of the 50 kHz calls may be of varying length, and sometimes, particularly the upper parts of the step calls, may be under angles. Frequency fluctuations are not to the scale. The right rubric provides exemplary references that show actual sonograms of similar types of vocalizations.

indicate that FL and FM 50 kHz vocalizations may be differentially regulated by the adrenergic system, thus these subtypes may reflect different emotive state. Effects of propranolol selectively suppressing FM 50 kHz calls signalling highly motivated situation might be relevant to some clinical reports that propranolol may decrease craving for cocaine and may have some anti-euphoric effect in human patients [131, 132].

The FM 50 kHz vocalizations could be further divided into those calls with trill or without a trill [104] (Fig. **1**). Trills, representing fast sin wave-like oscillations of the call frequency, were regarded as expression of the highest state of arousal and motivation [32]. Trills could be emitted alone or added to other call types usually step calls (Fig. **1**). It was demonstrated in a detailed pharmacological bioacoustic study that selective D1 receptor antagonist (SCH-23390) or nonselective competitive opioid receptor antagonist (naltrexone, with higher affinity to μ-opioid receptor than other opioid receptors) dose-dependently decreased FL 50 kHz calls as well as non-trill FM 50 kHz calls but had no effect on vocalizations with trills (i.e., step-trill or trill alone) [129]. It is possible, therefore, that trill-type vocalizations form a separate subcategory of 50 kHz vocalizations and be depended on a partially different neurochemical mechanism. The trill-type vocalizations may serve as a sensitive measure of high emotional arousal or intensity of positive affect because these types of calls were gradually decreasing with long-term self-application of cocaine and were most affected by brief periods of abstinence in addicted rats [133]. Moreover, repeated application of amphetamine could cause sensitization with increased emission of trill-type FM 50 kHz vocalizations but not FL 50 kHz calls [134], however, sensitization of trill-type calls was not reproduced, as tested for amphetamine, morphine or nicotine in a recent study [135].

PHARMACOLOGICAL STUDIES OF 22 kHz VOCALIZATIONS AND THEIR CLASSIFICATION

In a parallel research line, researchers have focused their attention to pharmacological agents that can evoke negative, aversive states signalled in rats by 22 kHz vocalizations. This line of research is relevant to interest in mechanisms of aversion, anxiety, behavioral inhibition, and negative affect, including mechanisms of many affective disorders in humans.

Intracerebral injection of cholinergic muscarinic agonists into a number of rostromedial mesencephalic, medial diencephalic and forebrain structures induced emission of abundant numbers of long 22 kHz calls as compared to vehicle injection [9, 23, 24, 59, 93, 136]. The vocal response was dose-dependent and antagonised by local pretreatment with equimolar doses of atropine or scopolamine [23, 136, 137]. There is no direct evidence pointing at a specific muscarinic receptor subtype that is responsible for the initiation of this response, however indirect evidence may point at M2 but not M1 muscarinic receptors. Decrease in locomotor activity and behavioral inhibition accompanying carbachol-induced response induced from the hypothalamus were reversed by M2 receptor antagonist (atropine) but not by M1 receptor antagonist (pirenzepine) [138]. On the other hand, strong activation of M1 muscarinic receptors in the hypothalamic-preoptic area of rats induced limbic-type of epileptic seizures [139], as well as, pirenzepine was also reported to prevent development of amygdala kindled seizures in rats [140].

Rat 22 kHz vocalizations have also shown some, although limited, acoustic heterogeneity. While call peak frequency (between 20 and 32 kHz) and bandwidth (1-6 kHz) showed limited changes across conditions and individuals, duration of single vocalizations was subjected to a substantial variability from very short calls (tens of ms) to over 3400 ms duration of a single vocalization [65, 68, 74, 75, 136] (Fig. **1**). Dose response study with intracerebral carbachol (dose rage of 0.24 - 4.0 μg) revealed more complex relationship between the intensity of the response and the duration of 22 kHz calls [136]. It was assumed that the higher the dose of the drug the more intensive the response. Increasing doses of carbachol induced, in general, shorter vocalizations with decreasing number of very long calls (over 1000 ms) and with an increasing number of short calls (less than 1000 ms in duration) [136]. Detailed distributions of 1000 vocalizations (200 calls per dose) revealed two peaks of call duration, at approximately 80-120 ms and 280-320 ms that were affected in a dissimilar way by the increasing doses of carbachol. This observation suggested that the 22 kHz vocalizations consist at least of two subpopulations of calls labelled short 22 kHz and long 22 kHz calls that were also observed in 22 kHz vocalizations induced by non-pharmacological, behavioral methods [68, 136]. It was concluded from distribution studies that short 22 kHz calls are those below 300 ms in duration and long calls those above that duration [68] (Fig. **1**).

The behavioral role of short 22 kHz vocalizations as compared to long calls is not entirely clear but they were abundantly observed during withdrawal from cocaine. Thus, it has been suggested that both short and log 22 kHz calls are associated with aversion [80]. It may be however suggested that short versus long 22 kHz calls express different states. A negative emotional state that is associated with an "internal" discontent (i.e., without an external danger or threat) is expressed by short calls, while the state caused by external danger is expressed by long calls.

In contrast to a variable single call duration, the 22 kHz peak frequency remained fairly stable between 20 and 30 kHz regardless of the method of their induction (hand touch, foot-shock, different doses of intracerebral carbachol, or injection of glutamate into the laterodorsal tegmental nucleus) [24]. The sound frequency is constantly maintained during the entire call with occasional "tuning" at the very first call of a series of vocalizations and a minor and non-specific frequency modulation caused by blood pressure fluctuations resulting from heart beat that do not carry coded information [24, 65, 141]. Also, the bandwidth was found to be narrow and stable, except some occasional initial fragment of downward frequency modulation that suggested that rats emit 22-kHz calls at the minimum possible ultrasonic frequency they can produce [65].

FUNCTION OF NEUROCHEMICAL SYSTEMS RESPONSIBLE OR INITIATION OF VOCALIZATIONS

The neural systems responsible for the initiation of two basic emotional states and emission of ultrasonic vocalization in rats were extensively reviewed in other publications [9, 24, 93, 142-144], so only a brief outline will be given in the present review.

Direct intracerebral pharmacological initiation of emotional responses with accompanying vocalization in rats enabled systematic mapping of receptive brain regions with quantitative assessment based on measurement of resulting vocalization. Amphetamine was used to map the positive state measured by 50 kHz vocalizations [20, 45] and carbachol was used to map the negative state measured by emission of 22 kHz alarm calls [136]. These studies led to identification of two anatomically and neurochemically different ascending brainstem projections originating from tegmentum, and with extensive target regions involved in the initiation of emotional/vocalization responses.

The mesolimbic dopaminergic system originating from the ventral tegmental area and travelling to the ventral striatum appeared to be the critical system for initiation of positive emotional state and emission of 50 kHz vocalizations [39, 143, 145]. The mesolimbic cholinergic system originating from the laterodorsal tegmental nucleus and travelling to the medial regions of the diencephalon, basal forebrain, and lateral septum was found to be responsible for the initiation of the negative emotional state and emission of 22 kHz vocalizations [9, 24, 93, 137, 143, 144, 146-149].

The relevant states and vocalizations could then be initiated from the brain in two ways. Firstly, pharmacological activation of the receptive fields with amphetamine for emission of 50 kHz calls or with carbachol for emission of 22 kHz calls could initiate a full positive or negative emotional state by activation of postsynaptic receptors: dopaminergic for positive response or cholinergic for negative response. Secondly, a nonspecific stimulation of neurons at the origin of these ascending pathways in the brainstem could also induce comparable emotional states and vocalization. Thus, electrical stimulation (that excites neuronal cell bodies and axons) of the ventral tegmental area induced 50 kHz vocalization [39], while chemical stimulation with glutamate (that excites all neuronal cell bodies) injected into the laterodorsal tegmental nucleus induced 22 kHz vocalizations [137]. These vocalizations induced from the source of the pathways did not significantly differ in acoustic parameters from those induced by chemostimulation of terminal receptive fields with dopaminergic or cholinergic agonists. Although, 50 kHz calls could also be induced by glutamate from the anterior hypothalamic-preoptic area, the response is still mediated by the dopaminergic system and could be antagonized by haloperidol [148].

Experimental damages to these ascending systems reduced or blocked the relevant responses and vocalization (dopaminergic system in rats [150, 151] and cholinergic system in cats [152]). Thus, activity of the mesolimbic dopaminergic pathway resulting with release of dopamine in the nucleus accumbens [42, 43] was responsible for the initiation of the positive state with emission of 50 kHz calls, while activity of the mesolimbic cholinergic pathway resulting with release of acetylcholine in the terminal fields of the diencephalon and forebrain was responsible for the initiation of the negative state with emission of aversive calls. The role of acetylcholine in the mesolimbic cholinergic pathway in rats was indirectly shown in an electro-physiological experiment [146] and in earlier pharmacological studies on cats [153].

The exact mechanism of the initiation of the emotional state with vocal response is not clear but it seems that the relevant initiating transmitters (dopamine or acetylcholine) need to be extensively released in the terminal target regions. These ascending projections from the tegmentum are part of the ascending reticular activating system that is generally responsible for arousal. We have recently suggested that the ascending cholinergic pathways from the laterodorsal tegmental nucleus represent a specific system for a negative emotional arousal [144], while the ascending dopaminergic pathway from the ventral tegmental area would form a specific system for a positive emotional arousal.

These two ascending emotional arousal systems seem to work by activation of reciprocal loops between their target areas and the midbrain limbic region, i.e., the origin of these projections, for the gradual initiation of the emotional state [149]. The evidence for such an organization came from c-Fos studies. Microinjection of carbachol into the hypothalamic-preoptic area (postsynaptic activation in relation to laterodorsal tegmental cholinergic neurons) induced, as expected, a strong negative state with 22 kHz vocalizations but, at the same time, the laterodorsal tegmental neurons (including cholinergic neurons) showed increased activity as evidenced by increased c-Fos labeling [149]. Only reciprocal connections from target areas back to the tegmentum would be responsible for such activation. Reciprocal circuit loops between the forebrain limbic areas and the midbrain limbic areas in the generation and maintenance of emotional states were originally suggested by Nauta [154] (for further details, see [149]). These loops are extensive [155] and may involve numerous transmitters as it was recently suggested for the laterodorsal tegmental nucleus and hypothalamic hypocretinergic and histaminergic transmitter systems that innervate the laterodorsal tegmental nucleus [156]. Hypocretin (orexin) and histamine were also shown to have effects on the ventral tegmental neurons [157, 158]. Serotonergic system is another example of reciprocal limbic midbrain – forebrain loops potentially involved in many emotional responses [159]. Further studies are needed to elucidate this complex mechanism for generation of emotional states, however, the ascending mesolimbic cholinergic and dopaminergic systems are the prime systems for the initiation of the emotional arousal and the resulting emotional state.

Pathological changes in functions of brain circuitries mentioned above will be also reflected in emitted calls. Thus, vocalizations emitted in positive and negative states serve not only as reliable indices of normal physiological conditions but also of pathophysiological processes and states. This offers a significant opportunity for studies of

Table 1. Brief list of milestones of neuropharmacological studies of ultrasonic vocalizations in the rat signalling emotional states.

Pharmacological and Related Findings	Agents Used	References
22 kHz ultrasonic vocalizations are induced from the rat hypothalamic-preoptic area by cholinergic stimulation.	carbachol, atropine	[23, 59, 136]
The magnitude of the 22 kHz ultrasonic response is dose-dependent and antagonized by muscarinic antagonist.	carbachol, atropine	[23, 136]
22 kHz vocalizations, induced by carbachol from septum and antagonised by antimuscarinic agent, are initiated by direct cholinergic input from the laterodorsal tegmental nucleus.	carbachol, atropine, scopolamine, glutamate	[24, 147]
22 kHz vocalization responses are mapped in the rat brain and form the medial cholinoceptive (responding to acetylcholine) vocalization strip along the neuraxis.	carbachol, atropine	[24, 93, 136]
22 kHz vocalizations induced from the laterodorsal tegmental nucleus by glutamate are antagonized by scopolamine injected into hypothalamic-preoptic area (part of the strip).	glutamate, atropine, scopolamine	[24, 137]
The ascending mesolimbic cholinergic system originating from the laterodorsal tegmental nucleus is postulated.	carbachol, scopolamine, glutamate	[146, 147]
Cholinergic neurons of the laterodorsal tegmental nucleus are active during emission of 22 kHz vocalizations.	carbachol	[149]
The mesolimbic cholinergic system that originates from tegmentum, induces negative emotional state. 22 kHz vocalizations signal negative emotional state.		[9, 24, 29, 79, 80, 89, 93, 144, 147
50 kHz vocalizations are induced by glutamate, but not carbachol, from the rat brain.	glutamate, carbachol	[148]
50 kHz ultrasonic vocalizations induced by intracerebral glutamate are antagonized by systemic haloperidol.	glutamate, haloperidol	[113]
50 kHz vocalizations are induced by amphetamine and the responses are mapped in the nucleus accumbens.	amphetamine	[20, 22, 45, 112]
Amphetamine-induced 50 kHz vocalizations are dose dependent and antagonized by dopamine antagonists.	amphetamine, raclopride, SKF-83566	[20, 45, 112]
50 kHz vocalizations are induced by other dopaminergic agents (given systemically or intracerebrally).	cocaine, methylphenidate, apomorphine, quinpirole	[21, 22, 110, 134]
50 kHz vocalizations are induced by release of dopamine in the nucleus accumbens from the mesolimbic dopamine system originating from the ventral tegmental area.		[9, 39, 42, 43]
Emission of the 50 kHz vocalization signals positive emotional state.		[9, 29, 32, 39, 40, 89, 125, 142, 143]
50 kHz vocalizations are divided into subtypes: flat calls, and frequency-modulated calls with steps and/or trills. The subtypes are postulated to have behavioral significance.		[28, 32, 104, 110, 124, 126]
Replay of 50 kHz vocalizations is self-administered by rats.		[32]
The system for negative emotional state signaled by 22 kHz calls and the system for positive emotional state signaled by 50 kHz calls are functionally antagonistic to each other.	carbachol, amphetamine	[9, 24, 143]
Positive or negative emotional arousal is initiated by two ascending reticular activating subsystems: mesolimbic dopaminergic subsystem or mesolimbic cholinergic subsystem, respectively.		[144]

The table summarizes main neuropharmacological and related studies of ultrasonic vocalizations in the rat that are associated with signalling negative and positive emotional states. Studies on other species are not included. The milestones of the studies are not necessarily organized in chronological order but reflect the progression of findings and ideas over the last 25 years. Main references indicate selected papers cited in this review only, and are neither organized in chronological order nor form a complete list. Many details of the studies were omitted, for example studies of receptor subtypes or many studies of acoustic details of the calls.

pathology of affective states in the rat models. Parameters of ultrasonic calls may also reveal developmental changes caused, for example, by prenatal exposure to cocaine [160, 161]. An extensive review of studies of affective disorders and addiction in the rat model is provided in this issue [162].

CONCLUSIONS

Ultrasonic vocalization evolved as highly adaptive intraspecies communication system from ancestral vocal communication of mothers with their offspring. Vocalizations always served for communication in biologically important situations and hence, were associated with emotional arousal. Significant emissions of ultrasonic calls are, therefore observed in emotional states and serve as indication of these states directed to conspecifics. Results of studies, and particularly pharmacological studies led to the conclusion that emission of rat ultrasonic vocalizations is an inseparable feature of their emotional states and it can serve as a reliable and sensitive index of these states. Based on the type of calls,

their number, and acoustic features, two basic emotional sates could be identified that are labeled as positive (appetitive) or negative state (aversive).

Positive emotional states are associated with emission of 50 kHz vocalizations and can be induced by appetitive and rewarding behavioral situations, as well as, by direct or indirect activation of the nucleus accumbens by dopaminergic drugs, including drugs of abuse. Emissions of 50 kHz vocalizations are mediated by D1, D2, and partially D3 dopamine receptors. On the basis of extensive literature review, three biologically significant subtypes of 50 kHz vocalizations could be identified, all expressing positive emotional states:

(1) flat calls without frequency modulation that serve as contact calls and have social coordinating function;

(2) frequency-modulated calls without trills (all types of step calls) that signal rewarding and significantly motivated situation;

(3) frequency-modulated calls with attached trills, or trills themselves, that are emitted in highly emotional situations associated with intensive affective state.

Negative emotional states are associated with emission of 22 kHz vocalizations that could be induced by aversive and dangerous situations, muscarinic cholinergic activation of cholinoceptive areas of medial diencephalon and forebrain, and are mediated by, at least D2 family of muscarinic receptors. Two biologically significant subtypes of 22 kHz vocalizations have been identified, both expressing negative emotional sates:

(1) long calls (more than 300 ms) that serve as alarm calls and signal external danger or potential danger;

(2) short calls that express a state of discomfort or distress without external source of danger.

The positive and negative states with emission of vocalizations are initiated by two ascending reticular activating subsystems: the mesolimbic dopaminergic subsystem originating from the ventral tegmental area, as a specific positive arousal system, and the mesolimbic cholinergic subsystem originating from the laterodorsal tegmental nucleus, as a specific negative arousal system. A brief summary of milestones of neuropharmacological studies of ultrasonic vocalizations in the rat signalling emotional states is presented in Table **1**.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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