# Comparison Between Cholinergically and Naturally Induced Ultrasonic Vocalization in the Rat

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**Ultrasonic vocalization in rats accompanying stressful situations or induced by direct brain stimulation may be used as a measure of emotionality and as a potential response model for testing anti-anxiety agents. The aim of the present study was to compare physical features of pharmacologically-induced ultrasonic vocalization with naturally triggered vocalization. Ultrasonic calls induced by hand touch, footshock, or by direct intracerebral injection of carbachol in adult rats were compared. Ultrasonic calls obtained in all these situations were described as '22 kHz' vocalization. Average frequencies of vocalization were 24.1 ± 0.78 kHz, 26.0 ± 2.64 kHz and 25.0 ± 1.87 (SD) kHz for handled, footshocked and carbachol injected rats, respectively, and they did not differ significantly from each other. Histograms of single call duration showed similar distribution patterns for all groups with a predominance of long calls, although carbachol-induced calls were shorter than calls induced by touch or footshock. Histograms for inter-call intervals showed one major peak at 100-150 ms for all groups. Sonograms and power spectra showed similar characteristics both for calls induced by intracerebral carbachol and by hand touch or footshock. The results indicate that physical features of ultrasonic vocalization induced by intracerebral carbachol are comparable with those for naturally induced vocalization and fall into the category of '22 kHz' calls.**

*Keywords:* ultrasonic vocalization, carbachol, intracerebral injection, vocal communication, sonogram, stress, anxiety, rat

## INTRODUCTION

Results of a number of studies indicate that ultrasonic vocalization of rats accompanying stressful or potentially dangerous situations may be used as a measure of emotionality and may serve as a basis for an animal model for testing anxiolytic agents (Gardner 1985; Tonoue et al 1986; Cuomo et al 1988; Eschalier et al 1988; Hard and Engel 1988; Kaltwasser 1990). Ultrasonic vocalization appears in young rats as a result of social isolation or as a result of such events as footshock or sudden acoustic stimuli applied to adult rats. Our recent studies (Brudzynski and Bihari 1990; Brudzynski et al 1991) have demonstrated that ultrasonic vocalization, which is comparable with the vocalization emitted in stressful situations, can be induced by acetylcholine agonists directly injected into the rat basal forebrain. This finding offers a possibility of inducing a controlled distress behavior without the presence of external stressors. Pharmacologically-induced aversive behavior with vocalization may be more useful for testing anti-anxiety agents than the naturally initiated responses because the measurable amount of vocalization is proportional to the emotional excitement (Brudzynski et al 1982), is dose-dependent (Brudzynski and Bihari 1990) so it can be easily controlled, and the vocalization is less sensitive to environmental influences.

Our recent studies attempt to demonstrate that cholinergically-induced ultrasonic vocalization reflects similar distress and has similar characteristics to vocalization triggered naturally by external stimuli. The aim of the present study was to compare physical features of ultrasonic calls

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induced by intracerebral carbachol with ultrasonic calls induced by footshock or by handling of naive rats in an unfamiliar laboratory environment.

## METHODS

### Animals

All procedures involving use of rats were approved by the University of Western Ontario Council on Animal Care and performed in accordance with the guidelines of the Canadian Council on Animal Care.

Thirty five rats weighing 250 - 450 g of b.w. were used in the study. Animals were kept in single cages with a 12:12 hr light/dark cycle and had standard pellet food and water ad libitum. Rats were taken for experiments during their early hours of the light phase, 3-5 days after arrival in the animal quarters.

### Experimental Design

Ultrasonic vocalization induced by intracerebral injections of carbachol was compared with vocalization induced by a 'mild' stressor (gentle touch with a human hand) or with vocalization induced by a 'strong' stressor, (electric footshock).

As indicated by our preliminary tests, approximately 60% of naive rats will vocalize to repeatable hand touch the first time in the laboratory. We therefore chose 9 vocalizing animals which will be referred to as the vocalizing to hand touch group. Another 9 rats were subjected to ten footshocks per session (footshock group). The remaining 17 rats had stereotaxically implanted bilateral cannulae in the anteromedial hypothalamic/preoptic area and were subjected to one or two unilateral injections of carbachol in a dose inducing vocal responses (carbachol group). All ultrasonic vocalizations emitted by rats in these three groups were recorded, analyzed and their physical characteristics were compared. In order to obtain a representative distribution of call durations or inter-call intervals for an 'average' stressful situation, the respective distributions obtained under 'mild' stress (hand touch) and 'strong' stress (footshock) conditions were pooled together.

### Hand Touch and Footshock Procedure

Naive rats were placed in a recording cage and were gently touched with a gloved hand in a repeatable manner usually to the nape of their necks for 10 min. The tactile stimulation was stopped as soon as the rat started to vocalize and was resumed when vocalization subsided for longer than 2 min.

Footshock was delivered by a shock generator (model SGS-003, BRS/LVE, Div. of Tech Serv, Inc., Beltsville, Md) through a floor grid of the recording cage. Rats received not more than 10 shocks per 10 min session (max. 1 mA, 1 s) and the sessions were repeated once per day for three days. Delivery of the footshock stimuli was stopped as soon as the rat started to vocalize and was resumed when the animal was silent for more than 2 min; however, the footshock was discontinued after 10 min or after 10 footshocks, whichever occurred first.

### Stereotaxic Surgery

Animals were anaesthetized with Ketamine (40 mg/kg i.m.)/Xylazine (3.2 mg/kg i.m.), placed in a Kopf stereotaxic apparatus, and bilaterally implanted with a stainless steel cannulae (640 $\mu$m, O.D.) into the anterior hypothalamic/preoptic area. The stereotaxic coordinates were: A = 8.7 to 7.7, L = 0.2 to 1.5, and V = 8.0 to 9.0 mm below the surface of the cortex according to the stereotaxic atlas by Paxinos and Watson (1986). Rats were given a one week recovery period. Out of 34 cannulae, ultrasonic vocalization was induced from 22 injection sites and their localization is illustrated in Fig. 1. Effective sites were mostly in the medial preoptic/anterior hypothalamic area and in the immediate vicinity of this region.

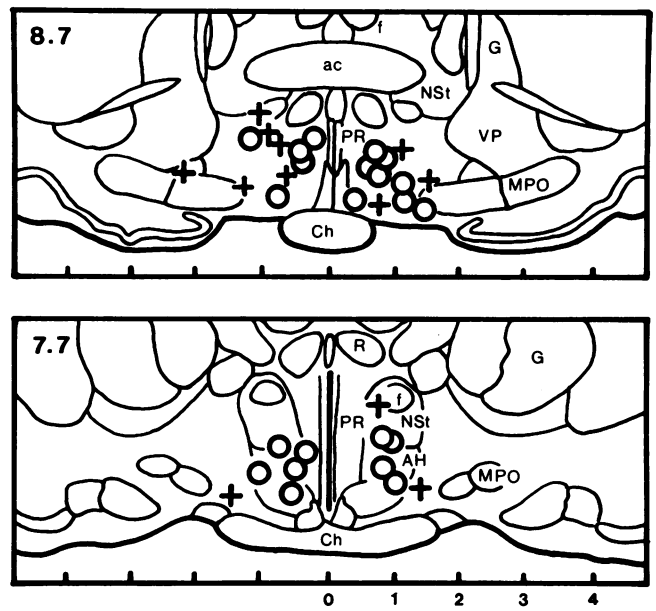


**Fig. 1. Localization of 34 injection sites (circles and crosses) within or in the vicinity of the anterior hypothalamic/preoptic area shown on the frontal sections of the rat brain (A = 8.7, upper and 7.7 mm, lower panel) according to the stereotaxic atlas (Paxinos and Watson 1986). Effective injection sites yielding ultrasonic vocalization are marked with circles, and ineffective sites with crosses. Abbreviations: ac - anterior commissure, AH - anterior hypothalamic area, Ch - optic chiasm, f - fornix, G - globus pallidus, MPO - magnocellular preoptic nucleus, NSt - bed nucleus of stria terminalis, PR - medial preoptic area, R - nucleus reuniens.**

### Drugs and Intracerebral Injection Procedure

Carbachol (carbamylcholine chloride, Sigma Chemical Co., St. Louis Mo.) was dissolved in sterile, pH controlled saline and was injected unilaterally into the brain by a Hamilton CR 700 microsyringe in a dose of 1.0 $\mu$g (5.47 nmole), in a volume of 0.2 $\mu$l, and at a rate of 4 nl/s. Injections of saline vehicle served as a control. For further details of surgery and microinjection technique see Brudzynski et al (1989).

### Recording and Analysis of Vocalization

Animals were tested in a padded, echo-free cage measuring 25 cm wide x 18 cm deep x 18 cm high. The observation cage was housed in a larger sound resistant, ventilated and temperature controlled cubicle. An ultrasonic microphone (model SM1, Ultra Sound Advice, London, England) was mounted in the center of the cage ceiling and connected to a S200 bat detector (QMC Instruments, Ltd, London, England). The frequency of emitted sounds was scanned from 20-50 kHz and the bat detector was tuned for zero beat frequency for each rat separately and maintained at that level for the 10 min recording period. Each call from the broadband output of the bat detector was amplified by a differential amplifier (Frederick Haer & Co., Brunswick, ME) and converted by an electronic circuit into a single square signal. The square signals were fed into a modified universal electronic counter (Intersil, model ICM 7226) to measure duration of ultrasonic calls and time intervals between them in seconds. The output from the counter was further fed into a personal computer in order to obtain frequency distributions from call durations and inter-call intervals. To compensate for non-vocalization ultrasonic noise, the same rats were placed in the experimental cage for 10 min a week later without tactile stimulation, footshock or carbachol injection and were left undisturbed. Ultrasonic vocalization did not occur and all recordings during these sessions were regarded as noise and were subtracted from the final distributions. Subtraction of distributions was done by a computer program in a bin by bin sequence. Negative results for some bins were shown on the graph as zero.

Signals from the bat detector were also analyzed on line by a DSP Sona-Graph signal analysis work station (model 5500, Kay Elemetrics Corp., Pine Brook, NJ) in order to obtain sonograms and power spectra from single calls or groups of calls and the results were printed on a Gray Scale Printer (model 5509, Kay Elemetrics Corp.).

## RESULTS

Injections of carbachol into the anterior hypothalamic/preoptic area induced emotional-aversive responses with ultrasonic vocalization. Injections of isotonic saline into the same brain sites were ineffective for all injection points.

Behavioral responses induced by repeatable hand touch, by electric footshock or by intracerebral carbachol were very similar to each other. Animals decreased their locomotor activity and resumed a crouched position in the cage. Ultrasonic vocalization appeared in the form of long calls with clearly distinguishable, long lasting 'pressure' expiratory movements. Vocalization appeared with a marked latency ranging from 1-60 s after hand touch and footshock and from 20-300 s after carbachol. Average frequencies obtained from the bat detector for n = 9 animals in each group (9 rats for carbachol were randomly chosen out of 22 effective responses) were basically the same among groups. They were 24.1 $\pm$ 0.78 kHz, 26.0 $\pm$ 2.64 kHz, and 25.0 $\pm$ 1.87 (SD) kHz for handled, footshocked and carbachol injected rats, respectively, and they did not differ significantly from each other (1-way ANOVA test, $F(2/24) = 2.74$, ns). Also the frequency ranges were comparable: 23-25 kHz for hand touch, 23-30 kHz for footshock, and 22-28 kHz for carbachol.

Distributions of single call duration are shown in Fig. 2. The distributions for hand touch (Fig. 2A), and in a lesser degree the distribution for footshock (Fig. 2B), showed two distinctive groups of calls ie., short and long calls. These two groups of calls were clearly seen in a combined distribution of calls from hand touch and footshock jointly (Fig. 2C). Distributions for naturally-induced calls in Fig 2 showed the range for short calls was approximately 40 - 300 ms and for long calls approximately 310 - 2000 ms. The distribution of call durations collected after injection of carbachol into 22 brain sites is shown in Fig. 2D. The overall range of call durations was shorter, ranging from 40 - 1500 ms; however, the distribution showed two similar groups of calls: short ones from approximately 40 - 200 ms and long ones from 210 to 1500 ms. The ranges for the two groups of calls were shorter by 25-30% as compared to those for hand touch and footshock.

Distributions of inter-call intervals are shown in Fig. 3. The intervals between single calls were distributed over a long span of time, but they formed a single group in the initial part of each histogram. The distributions for hand touch and for footshock (Fig. 3A,B) showed one dominating peak at approximately 100-150 ms. This peak is more clear when shown on a combined distribution for hand touch and footshock jointly (Fig. 3C). While the distribution for inter-call intervals for the hand touch group had a smooth, narrow peak at 100-110 ms, the distribution for footshock had a broader peak at 100-150 ms and a group of smaller accompanying peaks (Fig. 3B). The distribution of inter-call intervals after injection of carbachol, collected from the same responses as for call duration, is shown in Fig. 3D. The dominating peak value was approximately 100-150 ms and was accompanied by group of smaller peaks in close proximity of the main one. Although the main peaks are essentially comparable among all groups, the exact peak values and the overall pattern of the distribution makes the result after carbachol more similar to the distribution of calls after footshock than after hand touch.

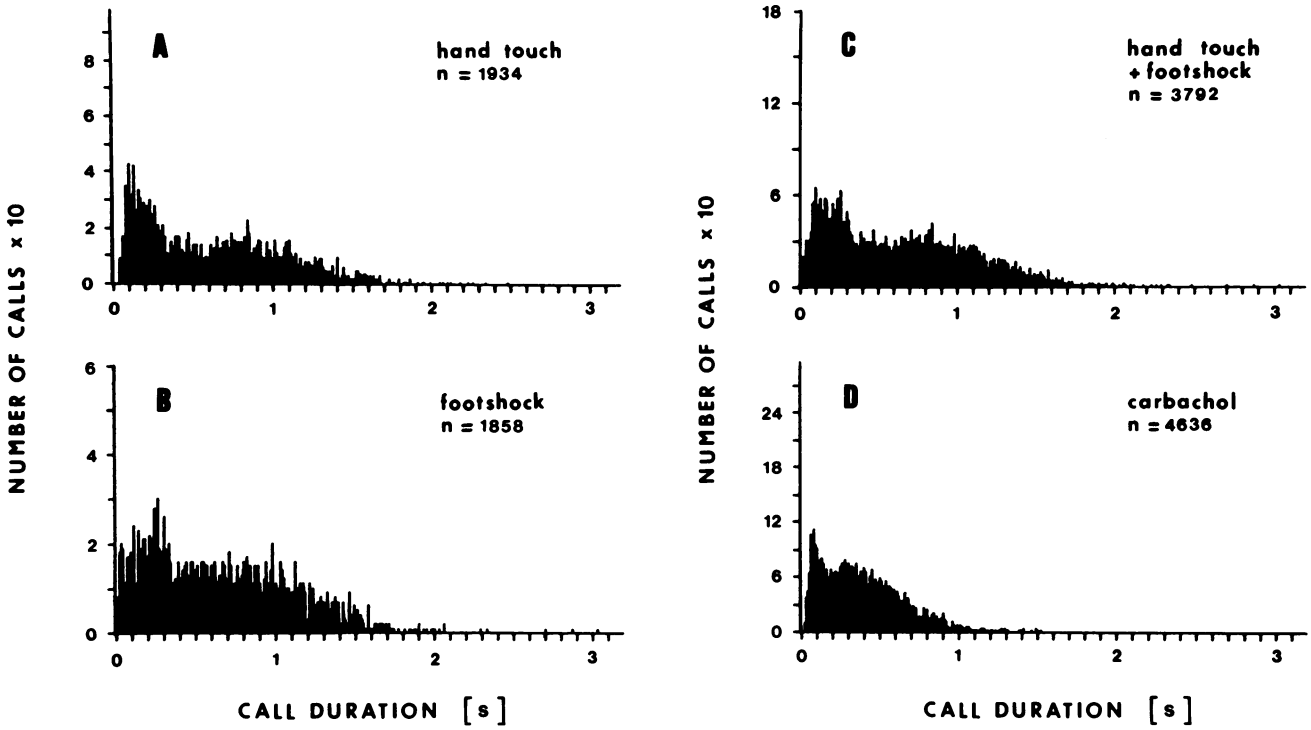


Fig. 2. Distribution of durations of ultrasonic calls induced by hand touch (A, 9 rats), footshock (B, 9 rats) and intracerebral injection of carbachol (D, 22 injection sites). The distribution in C was obtained by addition of the distributions in A and in B. The n values indicate the number of ultrasonic calls collected for each distribution. See Methods for further explanations.

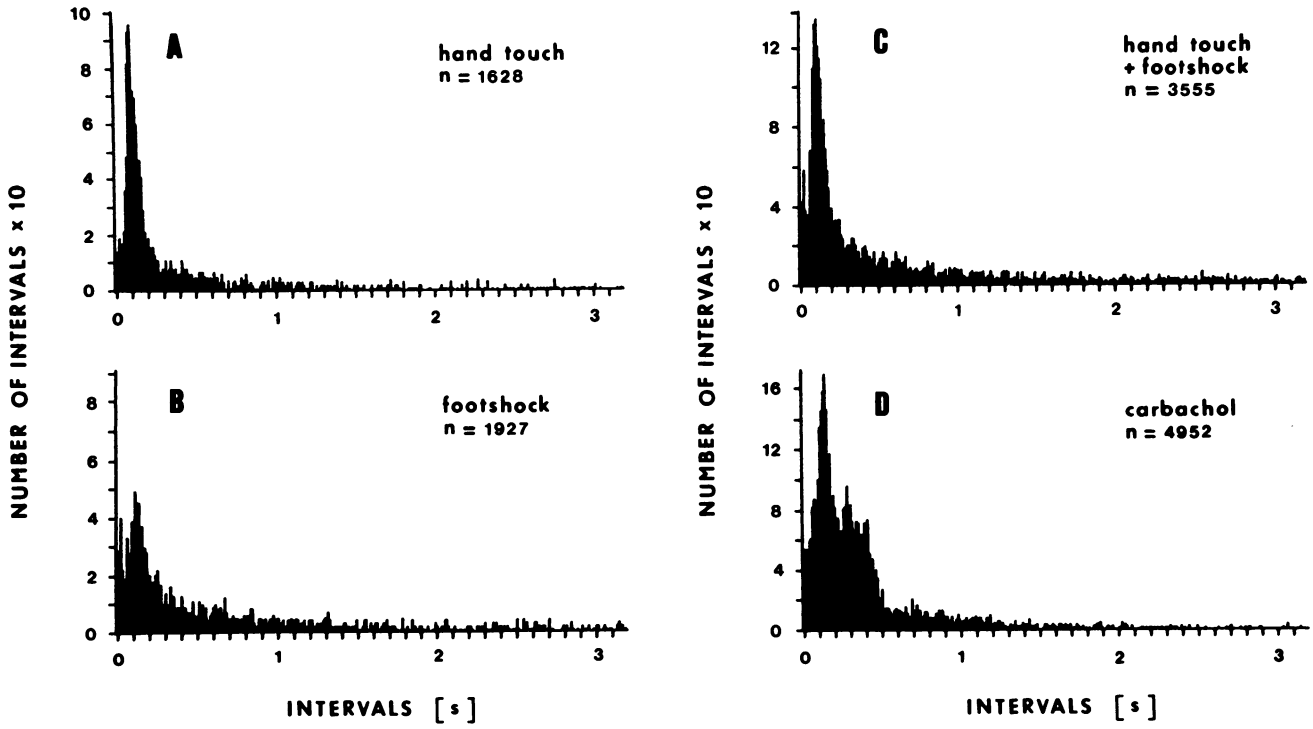


Fig. 3. Distribution of inter-call intervals from the same responses as in Fig. 2: for response to hand touch (A, 9 rats), to footshock (B, 9 rats) and to intracerebral carbachol (D, 22 injection sites). The distribution in C was obtained by addition of the distributions in A and in B. The n values indicate the number of inter-call intervals collected for each distribution. See Methods for further explanations.

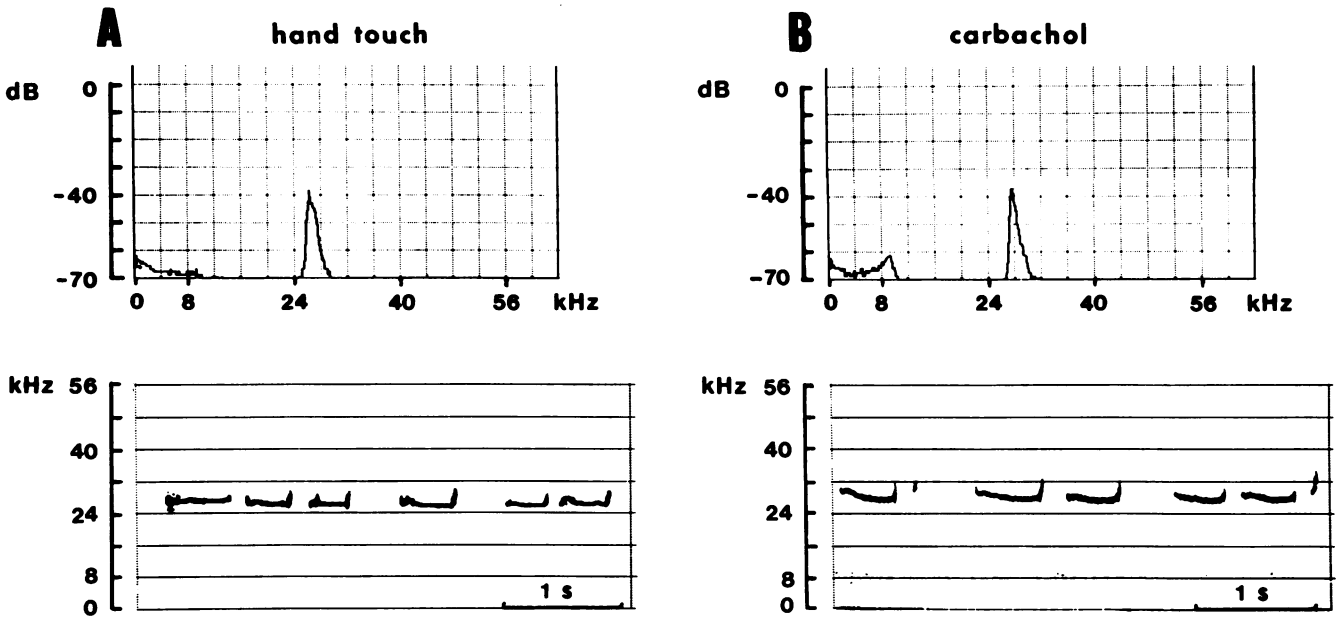


**Fig. 4. Power spectra (upper panels) and sonograms (lower panels) of a short series of ultrasonic calls induced by hand touch (A) or by intracerebral injection of carbachol (B). Power spectra were calculated jointly for all calls which are displayed on sonograms in lower panels. The power spectrum peak in A is at 26.52 kHz and in B at 27.68 kHz. Relative power of calls was calculated from the waveform displayed logarithmically in 100 ms steps, referenced from the top of the scale and expressed in negative decibels. Sound frequency is expressed in kHz.**

Finally, analysis of sonograms and power spectra for ultrasonic calls for all three groups did not show any significant differences among them. Examples of a sonogram and power spectrum for a group of ultrasonic calls induced by hand touch and by carbachol are shown in Fig. 4. Frequencies of calls, their time pattern and power spectra were comparable for all calls analyzed. The sonograms had also shown that bandwidths of all calls were similar and remained in a narrow range below 5 kHz (Fig. 4, lower panels).

## DISCUSSION

Ultrasonic vocalization induced by intracerebral application of carbachol showed essentially similar physical features to those of vocalization induced by a gentle hand touch or by electric footshock. Carbachol-induced vocalization had comparable average sound frequency and frequency range, distribution of call duration and inter-call intervals relative to respective parameters of vocalization induced by external stimuli. Analysis of inter-call intervals has further demonstrated that carbachol induced vocalization had than interval distribution more similar to that after footshock than to the distribution after hand touch.

Average frequencies of vocalization induced by hand touch, footshock or carbachol did not differ significantly from each other (24-26 kHz) and were contained within the range of so-called '22 kHz' vocalization (20-32 kHz, Sales and Pye 1974; Nyby and Whitney 1978; Sales 1979; Tonoue et al 1986). Both the carbachol-induced vocalization and hand touch- or footshock-induced vocalizations also had also other common features of the '22 kHz' vocalization, such as a single frequency component, narrow bandwidth (below 5 kHz) and predominantly long calls (70% of calls longer than 300 ms; Sales 1972; 1979; Barfield and Geyer 1975; Smith 1979; Tonoue et al 1986).

Analysis of the distributions of call duration also revealed that carbachol-induced vocalization had 25-30% shorter calls. This difference may be explained by the nature of the pharmacological stimulation. The presence of carbachol molecules in the vicinity of receptors for a time period of minutes may result in 'tonic' stimulation causing the animal to emit more calls per unit of time than under natural conditions, thus shortening the duration of individual calls. There is a second possibility that pharmacological stimulation releases a basic innate pattern of the physiological response which is not subjected to any external or internal modulations, and that such a basic pattern of the vocalizational response consists of shorter calls than normally emitted by adult rats. The observation that wild rats emit much shorter '22 kHz' calls than laboratory rats (up to 600 ms compared to over 3000 ms in laboratory rats; Sales 1972) and that there is a variation among different rat strains as to the length of the individual calls (Sales 1979), may corroborate this suggestion.

In spite of these differences, duration of the long '22 kHz' calls was reported to be equal to or longer than 300 ms as studied in two strains of laboratory rats and a strain of wild rats (Sales 1972, 1979). This observation is in agreement with a clear trough at approximately 200 - 400 ms on distributions of call duration in the present study, indicating that an approximate value of 300 ms may be regarded as a boundary between long and short calls.

The present results have shown that vocalization induced in the groups of rats in this study falls into the category of '22 kHz' calls, and that physical parameters of the ultrasonic vocalization induced by intracerebral carbachol are comparable with those for naturally induced vocalization. Thus, carbachol-induced vocalization can be used for measurement of emotional-aversive responses in a similar way to naturally induced calls.

## ACKNOWLEDGEMENT

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