The Role of the GABAergic System in the Production of Ultrasonic Vocalization in Rats

by

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Abstract

Ultrasonic vocalization plays an important role in intraspecies communication for rats. It has been well demonstrated that rats will emit 22kHz vocalization in stressful or threatening situations. Although the neural mechanism underlying vocalization is not well understood, it is known that cholinergic input to the basal forebrain induces such alarm calls. A number of experiments have found that intracerebral injection of carbachol, a predominantly muscarinic agonist, into the anterior hypothalamic/preoptic area (AH/POA) reliably induces vocalization similar to naturally emitted ultrasonic calls. It has also been shown that carbachol has extensive inhibitory effects on neuronal firing in the same area. This result implies that the inhibitory effects of carbachol in the AH/POA could trigger vocalization, and that the GABAergic system could be involved. The purpose of this study is to investigate the effects of GABA agonists and antagonists on the production of carbachol induced 22kHz vocalization. The following hypotheses were examined: 1) application of GABA (a naturally occurring inhibitory neurotransmitter) will have a synergistic effect with carbachol, increasing vocalization; and 2) the application of GABA antagonists (picrotoxin or bicuculline) will reduce carbachol-induced vocalization. A total of sixty rats were implanted with stainless steel guide cannulae in the AH/POA area. After recovery, animals were locally pretreated with 1) GABA (1-40µg), 2) picrotoxin (1.5µg) or bicuculline (0.03µg), or 3) saline; before injection with carbachol (1.5µg). The resulting vocalization was measured and quantitated. The results indicate that pretreatment with GABA or GABA antagonists had no significant effect on vocalization. Local pretreatment with GABA did not potentiate the vocal response as measured by its duration, latency, and total number of calls. Similarly, pretreatment with picrotoxin or bicuculline had no effects on the same measures of vocalization. The results suggest that cholinoceptive neurons involved in the production of alarm calls are not under direct GABAergic control.

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List of Abbreviations

Abbreviation	Term
ACh	acetylcholine
AChE	acetylcholinesterase
AH/POA	anterior hypothalamic/preoptic area
CCh	carbachol
CCK	cholecystokinin
cm	centimeter
ChAT	choline acetyltranseferase
GABA	γ-aminobutyric acid
ic	intracerebrally
ip	intraperitoneally
kHz	kilohertz
LDT	laterodorsal tegmental nucleus
ml	milliliter
mm	millimeter
ms	millisecond
NMDA	N-methyl-D-aspartic acid
PEI	postejaculatory refractory period
s	second
sc	subcutaneous
SN	substantia nigra
UV	ultrasonic vocalization
VTA	ventral tegmental area
µg	microgram
µl	microliter
µm	micrometer

Introduction

Ultrasonic vocalization plays an important role in intraspecies communication for rats. It has been well demonstrated that rats will emit 22 kHz vocalization in stressful or threatening situations. Because such vocalization usually only occurs in such situations, it may be used as a measure of emotional-aversive behaviour (Blanchard et al., 1990; Brudzynski et al. 1991a; Blanchard et al. 1991) and may facilitate studies of neural circuits involved in expression of emotionality. The central cholinergic system has been implicated in the production of ultrasonic alarm calls in adult rats (Brudzynski & Bihari, 1990; Brudzynski et al., 1991b). More specifically, pharmacological induction of these calls suggests that the ascending cholinergic pathways appear to initiate the response (Brudzynski & Barnabi, 1996) through widespread inhibitory effects on neuronal firing in the 'medial cholinoceptive vocalization strip" (Brudzynski et al., 1998a). Further, local injection of carbachol into the anteromedial hypothalamic preoptic area has reliably induced alarm calls in a number of studies. Carbachol-induced 22 kHz ultrasonic vocalization in the absence of external aversive stimuli produces alarm calls with acoustic characteristics which do not differ from naturally emitted calls (e.g., calls produced in response to stress or footshock) (Brudzynski et al, 1991b). This pharmacologically induced response presents a model that allows us to investigate the neural circuitry underlying the vocalization.

GABA (γ-aminobutyric acid) is a naturally occurring neurotransmitter that plays an essential role in the local control of neuronal excitability throughout the circuits of the central nervous system. It has a widespread and powerful inhibitory action on

neuronal activity. Interestingly, muscarinic inhibition (both natural and carbachol induced) has been postulated to trigger ultrasonic vocalization (Brudzynski, 1998). This presents the intriguing question of the role of the GABAergic system in local circuitries controlling ultrasonic vocalization.

The purpose of this study is to test whether GABA and/or GABA antagonists (picrotoxin or bicuculline) will affect carbachol-induced vocalization. Will GABA and carbachol have synergistic action or are their effects independent? The following hypotheses will be examined: 1) application of GABA will have a synergistic effect with carbachol, increasing vocalization; and 2) the antagonistic action of picrotoxin or bicuculline on GABA receptors will reduce carbachol-induced ultrasonic vocalization. It is important to know whether these hypotheses will be confirmed or rejected to better understand vocalization triggering circuitry. These mechanisms are relevant to effects of anxiolytic drugs like diazepam, whose action depends on modulation of the GABAergic transmission.

Overview

The communicative role of ultrasonic vocalization in rats

A number of studies have suggested that 22 kHz calls are communicative in nature, conveying important information for conspecifics. Frequently referred to as 'alarm cries' or 'alarm calls' (Blanchard, Blanchard, Agullana & Weiss, 1990; Brudzynski, 1998; Brudzynski & Barnabi, 1996: Calvino et al., 1996), the vocalization is produced in a number of behavioural settings including sexual behaviour, fighting behaviour, defensive behaviour or under stress (Brudzynski, 1994; Sales & Pye, 1974; for review see Miczek, Tornatzky, & Vivian, 1991). Calls observed in these situations range in sound frequency from 20-35 kHz. These calls are consistently produced in anticipation of unavoidable aversive stimuli. Direct application of carbachol to the anteromedial hypothalamic-preoptic area in the rat reliably induces 22 kHz vocalization (Brudzynski, 1994; Brudzynski & Barnabi, 1996; Brudzynski & Bihari, 1990; Brudzynski, Ociepa, & Bihari, F., 1991). As a result, it has been suggested that cholinergic input to the mediobasal forebrain and the diencephalon may contribute to the initiation and production of 22 kHz calls (Brudzynski & Barnabi, 1996). Hypothalamic and limbic structures are important in regulating behaviour and reproduction (Kupferman, 1991a). Hence, it is important to understand the underlying brain mechanism involved in the production of these calls.

The suggestion that ultrasonic vocalization (UV) can be studied as a measure of emotional-aversive behaviour is not without controversy. It has been suggested that 22 kHz calls may simply be a respiratory by-product of deep breathing, combined with

anatomical features of the animal's larynx (Blumberg & Alberts, 1990). This is based on the observation of interrelations of vocalization and physiological changes which are stated to occur simultaneously under stress conditions. For example, the onset of 22 kHz vocalization is generally accompanied with a decrease in locomotor activity (Brudzynski, McLachlan, & Girvin, 1989). It has been hypothesized that 22 kHz vocalization might be a by-product of deep expiratory respirations against a maximally constricted larynx. As this behaviour helps to cool the hypothalamus, it has been concluded that vocalization is influenced by the thermoregulatory state of the animal. This notion originated from the knowledge that the ultrasonic response of rat pups (50-54 kHz) typically occurs with cold exposure and appears related to pup's thermoregulatory abilities (Blumberg & Alberts, 1990). However, further investigation suggests that selective brain cooling is effected primarily by vasomotor changes within the nasal mucosa rather than the breathing maneuver. In addition, maximal ultrasound production occurs after one week postpartum when pups are better equipped to slow their rate of cooling before attaining homeothermy and are at less risk of death from cold exposure. Hence, it is inaccurate to say that rats depend solely on the vocalization for their survival in changed thermal conditions. Ultrasound production is widely considered to be a communicatory behaviour that elicits maternal retrieval to the warm nest (Blumberg & Alberts, 1990; Brudzynski, Kehoe, & Callahan, 1998; Insel, Hill, Mayor, 1986; Panksepp, Normansell, Herman, Bishop, & Crepeau, 1988). Emission of ultrasonic calls by pups in dangerous or potentially dangerous situations plays an adaptive role in activating the maternal response of locating and retrieving the vocalizing pup. This suggests that pup calls convey biologically significant information

for the mother, triggering an appropriate maternal response which increases the chances of pup survival.

In adult rats "alarm calls" may serve multiple purposes including the elicitation of predatory investigation, defensive or aggressive behaviour (Blanchard et al., 1990; Miczek et al., 1991). This is not so different from the theory of human emotion that suggests that similar physiological responses accompany different emotions (as reviewed by Kandel, Schwartz, & Jessell, 2000). It is only by assessing our environment that we attach a meaning to our physiological response (excitement versus fear). It is accurate that physiological differences between pups and adult rats can explain differences in vocalization characteristics. However, it seems overly simplistic to suggest that ultrasonic vocalization is incidental sound production without communicative value.

Further evidence to suggest the communicative value of UV in adult rats is the observation of slight variations of acoustic parameters in different behavioural settings. It has been postulated that the emission of short calls may be associated with aggressive motivation while longer pulses may relate to submissive motivation (Sales & Pye, 1974). Long calls may have a communicatory effect of behavioural inhibition.

More recent research has related differences in call morphology with the communicative value of 22 kHz calls (Van Der Poel & Miczek, 1991). Individual calls with initial segments consisting of downsweeps appear more often following defeat than following ejaculation or tail pinching. Modulated initial segments appear more frequently following ejaculation, while monotonous initial segments appear more often following tail stimulation. In addition, post-ejaculation calls are characterized by a

significantly larger proportion of calls that show frequency modulation compared to defeat and post-tail pinching calls. It has been suggested that frequency modulations observed in different behavioural contexts may reflect differences in the internal state of the animal. Further support of this is the observation of concurrent behavioural differences. During the post-ejaculation inactivity state a rat will attack an intruder, but an inactive defeated rat will respond with defensive or escape behaviours. In summary, it appears that at least two different types of 22 kHz calls exist, reflecting differing affective states: 1) a modulated call that may reflect a state of behavioural inhibition (as seen during post-ejaculation period, and 2) a monotonous call which may reflect a state of intense fear induced by physical threat. Both types of calls may be similar in that they convey a "desist-contact" message. Differences in the frequency modulation of calls based on the behavioural context may convey different messages whereas the temporal patterning is more likely the result of breathing capacity.

Further evidence to support the communicative nature of UV is from research which has found that rats vocalize when placed in a social situation but not when they are tested alone. Arthritic rats emitted UV prior to any physical contact with a healthy partner. In contrast, when exposed to a noxious stimulus, arthritic rats emitted audible squeaks (Calvino, Besson, Boehrer, & Depaulis, 1996). In their research examining 22 kHz calls in the rats in the visible burrow system, Blanchard et al. (1990) found that the presence of conspecifics significantly influenced the prevalence of vocalization. In addition, drug treatment suppressing 22 kHz vocalizations in male rats significantly affects the female's sexual behaviour, but not other activites such as self-care or exploration (Mos, Van Logten, Bloetjes, & Olivier, 1991). This suggests a specific

biological purpose as a warning signal to conspecifics to minimize contact, which in this context is likely to be painful or aversive.

The role of the cholinergic system in the production of ultrasonic vocalization

Intracerebral injections of carbachol, a predominantly muscarinic agonist, into the anterior hypothalamic/preoptic area (AH/POA) reliably induced emotional-aversive responses with ultrasonic vocalization (Brudzynski, 1994; Brudzynski et al., 1991a; Fu & Brudzynski, 1993). As a result it has been well accepted that cholinergic input to the mediobasal forebrain and the diencephalon may contribute to the initiation and production of 22 kHz calls. The term cholinergic indicates that neurons use acetylcholine (ACh) as a neurotransmitter (Kandel, Schwartz, & Jessell, 2000). In the brain, ascending components of the medial forebrain bundle distribute fibres to the hypothalamic and preoptic nuclei, with the longest of the fibers terminating in the septal region (Nauta, 1960; Moon-Edley & Graybiel, 1983). Basal forebrain areas have been shown to receive ascending cholinergic projections from the laterodorsal tegmental nucleus (LDT) (Satoh & Fibiger, 1986) with connections from tegmental pedunculopontine nucleus and parabrachial nucleus (Fulwiler & Saper, 1984; Moon-Edley & Graybiel, 1983) (see Figure 1). These areas are also known to contain cholinergic somata (see Figure 1) (Brudzynski, Kadishevitz, & Fu, 1998; Dencev, Hrycyshyn, & Brudzynski, 1996; Satoh & Fibiger, 1986).

Functional mapping of the vocalization response has identified an area which has been referred to as the 'medial cholinoceptive vocalization strip' (fig. 1) (Brudzynski, 1998). This strip includes 1) periventricular diencephalic areas, 2) a

limited region of the AH/POA (medial structures between 6.7-8.7 mm from the interaural plane) and 2) the area of the septum (structures rostral to 9 mm from the interaural plane) (Brudzynski, 1994; Dencev, et al., 1996). The combined areas of the AH/POA area and the septal areas appear to represent a well-localized continuous strip of the most responsive tissue from the anterior hypothalamus to the septum which receives cholinergic input involved in the initiation and production of ultrasonic vocalization (Brudzynski, 1994; Brudzynski & Barnabi, 1996). It is noteworthy that some research has found that the hypothermic effects of cholinomimetics are related to stimulation of the central cholinergic system (Kleinrok, Wielosz, & Poddubiuk, 1973). In summary, functional and anatomical studies have identified an ascending cholinergic pathway from the LDT which innervates the limbic and diencephalic structures and is critical in the control of behaviour and may also play an important role in the regulation of vocalization (Brudzynski & Barnabi, 1996; Brudzynski et al., 1998a).

In addition to identifying structures involved in ultrasonic vocalization, research has investigated the role of the ascending cholinergic projection from the pontomesencephalic cholinergic nuclei to the mediobasal AH/POA in the production of 22 kHz calls (Brudzynski & Barnabi, 1996). The distribution of sites effective in inducing vocalization by stimulation with L-glutamate coincided with that of LDT cells stained for acetylcholinesterase (AChE) and/or choline acetyltransferase (ChAT). ChAT is the transferase that is the characteristic enzyme in ACh biosynthesis. AChE is the enzyme responsible for rapidly hydrolyzing ACh (Kandel et al., 2000; Koelle, 1987). The presence of AChE and ChAT staining cells indicate a cholinergic nature of this nucleus. It has been previously established that most of the ascending LDT nucleus

projections are cholinergic (Satoh & Fibiger, 1986). Since the vast majority of LDT neurons have been shown to be cholinergic, glutamate had to activate at least some of the cholinergic neurons.

In addition, pharmacologically induced vocalization by application of L-glutamate to the LDT was attenuated or blocked by pre-treatment of scopolamine or atropine in the AH/POA. Glutamate is an amino acid with universal cellular constituents that functions as an excitatory neurotransmitter. Scopolamine and atropine are cholinergic antagonists which block cholinergic receptors. Blockage of glutamate-induced vocalization with application of a muscarinic antagonist in the AH/POA is further evidence for an ascending ACh pathway from the LDT to the AH/POA, and its role in the production of calls. CCh-induced vocalization is pharmacologically specific and has not been induced by injection of vehicle alone or drug pretreatment. Finally, CCh application which produces 22kHz vocalization is confined to areas receiving cholinergic innervation from the LDT. A substantial portion of the mesopontine cholinergic projection terminates in the AH/POA.

Support for the 'medial cholinoceptive vocalization strip' from diffusion studies

Pharmacological induction of ultrasonic vocalization from a well localized 'medial cholinoceptive vocalization strip' is further supported by evidence from diffusion studies. Microinjection of CCh into the preoptic and anterior hypothalamic areas (particularly the medial preoptic nucleus) consistently elicits 22 kHz vocalization accompanied by a marked decrease in spontaneous locomotion (Brudzynski McLachlan, & Girvin, 1989). The decrease in locomotion is dose dependent and

reversed by an equimolar dose of atropine, suggesting that the motor effects of CCh are anatomically specific and that a decrease in locomotion can be elicited from a limited forebrain area. However, this conclusion is valid only if the delivery of CCh is confined to the targeted area (Myers, 1974). Of major concern is whether the CCh exerts its effects in the AH/POA or some secondary locus. It is possible that the drug might spread one of three ways: ventricular transport, local blood circulation or diffusion through the interstitial fluid of the brain tissue. It is generally recommended that the volume injected into the brain of the rat should not exceed 0.5 µl (Myers, 1974). Microinjection of 1 µl of radioactive carbon-labeled morphine into the hypothalamus of the rat is retained within 1 mm from the injection site (i.e., within a 2 mm diameter area) (Lomax, 1966). In addition, microinjection of different dyes into the hypothalamus in a volume range of 0.5 to 3µl do not reach the ventricles, with only marginal spread to cerebrospinal fluid (Myers, 1974). Behavioural studies indicate that direct injection of CCh into the ventricular system does not induce locomotor or other behavioural changes elicitable from basal forebrain and hypothalamic structures despite being transported throughout the ventricular lumen. Hence, in the unlikely event that CCh reached the ventricle it would not likely produce a behavioural effect. In addition, no correlation has been found between the magnitude of the CCh effect on locomotion and the distance of the injection site from the ventricle wall (Brudzynski, et al., 1989).

In diffusion studies, injections of radiolabeled compounds into the hypothalamic area revealed an area of spread comparable with microinjections of dyes forty minutes after injections. This suggests that the effective dose of a drug is retained within the injected structure. In CCh-induced vocalization studies behavioural data collection

begins immediately following drug injection and continues for 10 minutes. As a result the diffusion area for CCh is likely to be less than those observed in diffusion studies. Pressure injection of CCh into the AH/POA at a comparable dose and volume (1.0 µg, 0.2 µl) caused a significant effect on neuronal firing within a 600 µm area (Brudzynski et al., 1991a). Further support that CCh remains in the target area is the evidence indicating that two adjacent areas induce opposite effects on locomotion following CCh injection. If CCh diffused from the target area, an increase in motor activity would be observed.

It is not likely that CCh is transported through local blood circulation. Even with the assumption that a percentage of CCh enters the blood, CCh reaching blood vessels would be eliminated from the brain since it does not cross the blood-brain barrier back into the neurons (for discussion see Brudzynski et al., 1989).

Historically, there has been some concern with the use of 0.9% saline as the vehicle for drug injections. Physiological saline may have independent effects on nerve tissue or may interact with a given drug. This is not considered to have an important role in this experiment, as injections of isotonic saline into the AH/POA have been ineffective in inducing ultrasonic vocalizations. Injections of carbachol into the same area induced emotional-aversive responses with ultrasonic vocalization for all injection points (Brudzynski, Ociepa, & Bihari, 1991).

Communicative value of pharmacologically induced calls

To be useful in the study of ultrasonic vocalization, pharmacologically induced calls must retain their communicative value. There is evidence that this is the case as

some studies indicate that adult rats will respond to the replay of pharmacologically induced calls (Brudzynski & Chiu, 1995). In addition, rats respond behaviourally to carbachol induced 22 kHz vocalization similarly to those produced naturally (Brudzynski & Chiu, 1995). Lastly, vocal response as measured by latency, response duration, and total number of calls does not differ between pharmacologically induced and naturally emitted calls.

Pharmacologically induced 22 kHz vocalization is typically accompanied with reduced locomotor activity. Similarly, a decrease in locomotor activity has been observed following the presentation of 22 kHz calls produced by either tactile stimuli or CCh injection, suggesting that effects caused by CCh-induced calls do not differ from those caused by naturally emitted calls. Hence, their communicative value remains the same (Brudzynski & Chiu, 1995; Brudzynski & Ociepa, 1992; Brudzynski et al., 1991b). Some specific observable behaviours characterize this decrease in motor activity. During vocalization bodies (sides or backs) are typically in contact with the cage wall while the animal remains motionless, heads are lowered and protruded, with breathing characterized by deep pressure exhalations. The animal's locomotion is substantially decreased with no clear escape attempts observed. The decrease of exploration and the onset of vocalization have been described as the most striking features of the rats' behaviour following injection of CCh into the AH/POA. Animals vigorously exploring the cage after CCh injection do not vocalize. Behavioural responses during vocalization do not differ qualitatively with dose of CCh, with animals showing a consistent pattern of behaviour. There does appear to be a relationship between dose and decrease in spontaneous locomotion.

In addition, higher doses of carbachol are related to a greater relative sound intensity. Pharmacologically induced vocalization is fully comparable with naturally triggered vocalization in the following parameters: sound frequency, narrow bandwidth, predominantly long calls (> 300ms in duration), and relative constant sound frequency (Brudzynski et al., 1991a). Average frequency of vocalization, does not differ significantly for handled, footshocked or carbachol injected rats. Single call durations show similar distribution across groups except that carbachol-induced calls are shorter (may be the result of pharmacological stimulation: not subjected to external or internal modulations). CCh-induced calls have similar inter-call intervals and similar acoustic characteristics. In summary, injection of CCh into the AH/POA produces 22kHz calls with similar vocalization and behavioural characteristics to natural behaviour. Hence, carbachol induced vocalization can be used as measurement of emotional aversive responses and compared with naturally produced calls.

Association of GABA with the production of ultrasonic calls

The amino acid γ-aminobutyric acid (GABA) has an inhibitory function in the central nervous system (Fonnum, 1987; Kandel, Schwartz & Jessel, 2000, Roberts, 1987, Simmonds, 1987). Although glycine also functions as an inhibitory neurotransmitter, it is mainly confined to the medulla and the spinal cord while GABA exerts its influence throughout the CNS (Fonnum, 1987). Neurotransmitter analysis indicates a large amount of GABA in the hypothalamus. GABA neurons play important roles in control mechanisms within the hypothalamus (Roberts, 1987). It has been suggested that GABA may have an important function in the neural control of sexual

behaviour in the rat (Fernández-Guasti et al., 1986). Injection of GABA antagonists stimulate sexual behaviour and shorten the postejaculatory refractory period (PEI). Conversely, GABA agonists depress sexual behaviour. Male rats emit 22 kHz vocalization during the PEI following ejaculation. Injection of (+)-bicuculline methiodide, a GABA antagonist, shortens the PEI period and reduces vocalization, reflecting a depression of an inhibitory state naturally produced by ejaculation (Fernández-Guasti et al., 1986). A highly significant reduction of vocalization occurred in rats treated with the GABA antagonist. This is evidence for a GABAergic role in the production of ultrasonic vocalization in rats.

Further research has implicated central GABA systems in the stress response of rats, particularly the $GABA_A$ receptor subtype (Hawkins et al., 1999). Central injections of the $GABA_A$ agonist muscimol increased stress related behaviour (food gnawing, revolution, number of vocalizations) in response to tail pinch. The number of vocalizations was increased significantly by tail pinch with a 100 ng dose of muscimol (but not 30ng) and not at higher dose of 300 ng. This indicates that activation of central $GABA_A$ receptors augments responses evoked by tail pinch, when injected into the cerebral ventricles, the substantia nigra (SN) or 1.0 mm anterior to the SN. Differences in latencies and effective dose were dependent on injection site, leading to the speculation that the GABA agonist (muscimol) was not acting within the SN but diffusing to other receptors in the ventral mesencephalon. Consistent with earlier discussion, the suggested site of action is the VTA, due to the knowledge that GABAergic mechanisms modulate dopamine systems arising in the VTA, the relationship between the VTA and the stress response and the proximity of the VTA to

the injection sites (Hawkins et al., 1999). These studies provide evidence for the role of the GABAergic system in the production of 22 kHz calls in sexual behaviour and tail pinch induced pain response.

Hypotheses

It has been well accepted that cholinergic stimulation of the AH/POA induces 20-30 kHz UV in adult rats. Neurons in this area respond to CCh application with a decrease in mean firing rate. GABA is a major inhibitory transmitter which may decrease mean firing rate. Injection of a GABA antagonist into the AH/POA has been found to reduce UV in rats (Fernández-Guasti et al., 1986). Carbachol-induced 22 kHz vocalization may present a model to investigate the role of the GABAergic system in the production of 22 kHz vocalizations in rats. As GABA exerts its effects on the central nervous system with widespread inhibitory effects it is hypothesized that 1) application of GABA will have a synergistic effect with carbachol, increasing vocalization; and 2) the antagonistic action of picrotoxin or bicuculline on GABA receptors will reduce carbachol-induced ultrasonic vocalization.

METHODS AND MATERIALS

Overview

Six series of experiments were conducted, with ten rats in each series. Following surgical implantation of two guide cannulae into the AH/POA, animals received two sets of injections one week apart (groups 'a'). To explore the role of GABA in the production of 22 kHz calls, groups 1-5 received a second set of two injections one week apart (groups 'b') with slight procedural variations (e.g., drug, dose, or cannula). Group 6 received identical procedures in both 'a' and 'b' experiments. Each set of injections included an injection of CCh (1-1.5 μg) preceded by a pretreatment injection of saline (control condition) or drug (experimental condition). Each animal received both treatments in counterbalanced order. Following injection, animals were placed in the recording chamber and vocalizations were recorded. Prior to experimentation animals were handled to eliminate UV elicited by human touch. All groups received one sham injection prior to commencement and at the conclusion of experiments. Group 6 received additional pre-exposure to the recording chamber. Histological preparations were analyzed under microscope and a map of injection sites was composed to verify accurate cannulae placement.

Animals and Surgery

All procedures involving the use of rats were approved by Brock University Animal Care Committee and performed in accordance with the guidelines of the Canadian Council on Animal Care. For a full review of surgical procedure, refer to Cooley & Vanderwolf (1977).

The study was performed on 60 male Wistar rats (Charles River, Montreal, Quebec) weighing 240-390 g at the time of surgery. All animals were housed in individual cages and allowed free access to Purina Rat Chow and water in a light controlled room with a 12-h: 12-h light/dark cycle and constant room temperature (21-23°c). Prior to surgery rats were given analgesic of buprenorphine (0.03 mg/kg ip, Buprenex, Reckitt and Colman Products, Hull, England), and ketamine hydrochloride (50 mg/kg ip, Ketalean, MTC Pharmacuticals, Cambridge, ON), and xylazine hydrochloride (3.2mg/kg ip, Rompun, Bayvet Div. Chemargo, Ltd., Etobicoke, ON) and placed in a Kopf stereotaxic apparatus for surgery. Ear bars were coated with the topical anesthetic lidocaine hydrochloride (20 mg/ml, Xylocaine Viscous 2%, Astra Pharma Inc., Mississauga, ON). The incisor bar was positioned 3.3 mm below the ear bar plane and stainless-steel guide cannulae constructed from 23-gauge hypodermic needle tubing were implanted bilaterally into the AH/POA. Stereotaxic coordinates for the guide cannulae, according to the Paxinos and Watson sterotaxic atlas (1986), were A = 8.1 mm anterior from the interaural plane, L = 0.6 mm to the midline, and V = 7.1 mm below the surface of the cortex for AH/POA. Guide cannulae were inserted into the brain 1mm above the target location and secured to the skull with jewelers stainless steel screws and dental acrylic (Perm, Hygenic Co., Acron, OH). The guide cannulae were temporarily closed using sterile wire pins to prevent blockage or the introduction of foreign particles to the brain. Animals received saline (3 ml, sc) post-surgery to prevent dehydration and enhance elimination of anesthetic. Animals were given a one-week recovery period, during which handling occurred to familiarize rats with the testing conditions and eliminate handling-induced stress. All groups received sham

injections prior to and upon completion of experiments, during which the injecting cannula was inserted into the guide cannula. Consistent with actual injections, the injecting cannula remained in position for 20s, when it was removed and plug pins were reinserted into guide cannula. Animals were subsequently placed in the recording chamber. None of the animals vocalized following sham injections. In addition, animals in group 6 were habituated to the recording chamber for ten minutes on three separate occasions, three days apart.

Drugs and Injection Procedure

All drugs were dissolved in 0.9% pyrogen-free, sterile saline (pH 6.5-7.0) and injected unilaterally into the brain by a 30-gauge stainless steel cannula. All injections were delivered via stainless-steel cannula connected by PE-10 polyethylene tubing (Clay Adams, Parsippary, NJ) to a CR-700 microliter syringe (Hamilton Corp., Reno, Nevada). The injection cannula extended 1.0 mm beyond the guide cannula so that it targeted the brain site precisely. Sterile, pyrogen-free 0.9% saline was injected into the same brain sites as a control. All drugs were injected at a rate of 0.2 ul/10 s. Animals in the control condition received an injection of carbachol (CCh, carbamylcholine chloride, Sigma Chemical Co., St. Louis, MO) into the AH/POA preceded by injection of saline into the same cannula. Animals in the experimental condition received an injection of CCh into the AH/POA preceded by GABA (1-40 μg), +-bicuculline or picrotoxin (as described below). All pretreatment injections were done 2-5 min before CCh injection. Injections of saline and drug pretreatment were counterbalanced with half of the animals receiving saline and half receiving the drug as a pretreatment. After

a drug injection, the injection cannula was left in position for 20s to prevent backflow of drugs upon removal and the guide cannula was sealed with a wire pin. Rats were then placed immediately into the recording chamber.

Experiment 1a: GABA (1 µg, 0.2 µl).

Animals in Group 1a received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1.5 µg, 0.2µl) into the AH/POA preceded by injection of saline (0.2 µl) into the same area; (2) injection of CCh (1.5 µg, 0.2µl) into the AH/POA preceded by injection of GABA (1 µg, 0.2µl). Pretreatment injections were done 5 minutes before CCh injection. Injections of saline and GABA were counterbalanced with half of the animals receiving GABA and half saline as their first injection.

Experiment 1b: Picrotoxin (0.2 µg, 0.2 µl).

Animals in group 1a were administered the same procedure one week later using a GABA antagonist instead of GABA. Animals in Group 1b received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1.5 µg, 0.2µl) into the AH/POA preceded by injection of saline (0.2 µl) into the same area; (2) injection of CCh (1.5 µg, 0.2µl) into the AH/POA preceded by injection of picrotoxin (0.2 µg, 0.2µl). Pretreatment injections were done 5 minutes before CCh injection. Injections of saline and picrotoxin were counterbalanced with half of the animals receiving picrotoxin and half saline as their first injection.

Experiment 2a: GABA (2 μg, 0.2 μl) pre-treatment.

Animals in Group 2a received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of saline (0.2 μl) into the same area; (2) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of GABA (2 μg, 0.2μl). Pretreatment injections were done 5 minutes before CCh injection. Injections of saline and GABA were counterbalanced with half of the animals receiving GABA and half saline as their first injection.

Experiment 2b: GABA (2 μg, 0.2 μl) pre-treatment.

Animals from Group 2a were reinjected one week later with an increased dose of GABA (5μg). Animals in Group 2b received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of saline (0.2 μl) into the same area; (2) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of GABA (5 μg, 0.2μl). Pretreatment injections were done 5 minutes before CCh injection. Injections of saline and GABA were counterbalanced with half of the animals receiving GABA and half saline as their first injection.

Experiment 3a: Bicuculline (0.3 μg, 0.2 μl) pre-treatment.

Animals in Group 3a received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of saline (0.2 μl) into the same area; (2) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of bicuculline (0.3 μg, 0.1μl). Pretreatment injections were done 5 minutes before CCh injection. Injections of saline and bicuculline were

counterbalanced with half of the animals receiving bicuculline and half saline as their first injection.

Experiment 3b: Bicuculline (0.3 μg, 0.2 μl) pre-treatment.

Animals in group 3a were reinjected one week later as group 3b. As no significant results were obtained in group 3a, the time period between pretreatment and CCh injections was reduced from five to two minutes. All other conditions in group 3b were identical to those in 3a. Animals in Group 3b received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of saline (0.2 μl) into the same area; (2) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of bicuculline (0.3 μg, 0.1μl). As previously stated, pretreatment injections were done 2 minutes before CCh injection. Injections of saline and bicuculline were counterbalanced with half of the animals receiving GABA and half saline as their first injection.

Experiment 4a: GABA (20 μg, 0.2 μl) pre-treatment.

Animals in group 4a received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of saline (0.2 μl) into the same area; (2) injection of CCh (1.5 μg, 0.2μl) into the AH/POA preceded by injection of GABA (20 μg, 0.2μl). Consistent with the previous group, pretreatment injections were done 2 minutes before CCh injection. Injections of saline and GABA were counterbalanced with half of the animals receiving GABA and half saline as their first injection.

Experiment 4b: GABA (20 µg, 0.2 µl) pre-treatment.

The same animals in group 4a were reinjected as group 4b with all conditions remaining identical.

Experiment 5a: GABA (40 µg, 0.2 µl) pre-treatment.

As no significant results were found in previous groups using lower doses of GABA, the dose was increased to 40 µg. In addition, the CCh dose was reduced to 1 µg. All other experimental conditions remained the same. Animals in Group 1a received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1 µg, 0.2µl) into the AH/POA preceded by injection of saline (0.2 µl) into the same area; (2) injection of CCh (1 µg, 0.2µl) into the AH/POA preceded by injection of GABA (40 µg, 0.2µl). Pretreatment injections were done 2 minutes before CCh injection. Injections of saline and GABA were counterbalanced with half of the animals receiving GABA and half saline as their first injection.

Experiment 5b: GABA (40 µg, 0.2 µl) pre-treatment.

The same animals in group 5a were reinjected as group 5b. All testing conditions remained identical, except that injections were administered into the right cannula.

Experiments 6a and 6b: GABA (40 µg, 0.2 µl) pre-treatment.

Animals in group 6a and 6b received the same injection procedure as group 5a. All injections were administered to the left cannula. Animals in Group 6a received a total of four injections during two sessions 1 week apart: (1) injection of CCh (1 µg, 0.2µl) into the AH/POA preceded by injection of saline (0.2 µl) into the same area; (2) injection of CCh (1 µg, 0.2µl) into the AH/POA preceded by injection of GABA (40 µg,

0.2µl). Pretreatment injections were done 2 minutes before CCh injection. Injections of saline and GABA were counterbalanced with half of the animals receiving GABA and half saline as their first injection. Animals in group 6 were habituated to the recording chamber prior to commencement of the experiment.

Recording and Analysis of Vocalization

Vocalization was recorded in a padded, echo-free experimental cage measuring 25 cm wide X 18 cm deep X 18 cm high. The cage contained an ultrasonic microphone with a working range of 10-180 kHz (model SM1, Ultra Sound Advice, London, England), wall mounted 2 cm above the floor and connected to a S200 bat detector (QMC Instruments Ltd, London, England). The bat detector was set for broad band recording with the frequency division ratio 1:16. The initial ten-minute vocalization period was recorded on a tape recorder and further analyzed by sonograph (DSP Sona-Graph signal analysis work station model 5500, Kay Elemetrics Corp., Pine Brook, NJ) to capture acoustic characteristics of calls. Response latency was recorded in seconds from time of replacement of the plug-pin post injection to the first vocalization. Response duration was measured from onset of vocalization to the last vocalization after which the rat had not vocalized for one full minute. Sonographic displays allowed for a detailed analysis of call sound frequency, call duration and bandwidth. The first 20 calls from each rat in each condition were analyzed from the tape recorder. Duration of individual calls was measured by manually placing sonograph cursors at the beginning and end of each call's sonogram (see Fig. 2, lower graph). A power spectrum was produced for each call and the highest frequency component was measured as peak

frequency. The bandwidth was obtained by measuring the frequency difference between lowest and highest sound frequency of the spectrogram peak.

Upon completion of the experiments, all injection sites were injected with 0.1μl of 2:1 diluted suspension of Indian ink. The rats were sacrificed by an overdose of sodium pentobarbital and perfused transcardially with 60-120 ml of 10% buffered formalin. The brains were subsequently removed from the skull and fixed in a 10% formalin solution.

Histological Verification

Coronal sections of 40 to 60 um were taken using a freezing microtome (Hacker Instruments Inc., Fairfield, NJ). The sections were mounted on gelatinized prepared glass slides, air dried, and stained with thionin for histological verification of injection sites. Maps of injection sites were composed with the aid of a projection microscope. Histological preparations were analyzed under microscope for verification of injection sites determined on the base of the location of the India ink deposit. A map of injection sites was composed according to the coronal brain sections from the Paxinos and Watson's (1986) stereotaxic atlas.

Research Design and Procedures

Experiments were conducted on six groups of ten rats per session. Using a within subjects design, animals in each group received a control pre-treatment and an experimental pre-treatment in counterbalanced order, with half the rats first receiving the control or experimental pre-treatment. Pre-treatment was followed with injection of

CCh (1-1.5 μg) dissolved in saline in all rats. Animals received the remaining treatment one week later. In the control condition, pre-treatment consisted of saline (0.2 μl) followed 2-5 minutes later by CCh injection. In the experimental condition, pre-treatment consisted of GABA (1-40 μg dissolved in 0.2 μl saline), picrotoxin (0.2 μg dissolved in 0.2 μl saline) or bicuculline (0.3 μg dissolved in 0.1 ml saline). All injections were given into the same injection site (typically the left injection cannula). Some groups (1 and 6) received injections to both brain sites, but a second site was used only after completion of a full set of injections in the first site and with one week (5-7 days) between injections. A time period of one week separated injections, during which time rats were weighed and handled every second day. Handling included imitation of the injection procedure to familiarize animals with the experimenter and experimental conditions. As seizure activity may interfere with UV animals displaying epileptic seizure behaviour were eliminated from future experiments. Similarly, rats ceasing vocalization after multiple injections were also eliminated from future experimentations to insure cessation of vocalization was not the result of nerve tissue damage.

Statistical Tests

Effects of drug pretreatment for each group were evaluated by a Student's t-test for paired samples. Differences in groups receiving similar treatment (i.e., Group 5 and 6) were assessed using a two-factor ANOVA. All statistical procedures were completed with SPSS program.

RESULTS

Injection of 1-1.5 μg of carbachol into the AH/POA consistently induced long alarm calls in most naïve rats (eight out of ten rats vocalized in groups 1-6, with the exception of group 2:10 vocalizing rats, and group 6: 9 vocalizing rats). Carbachol induced vocalization fell into the category of 22 kHz alarm calls in all animals (20-32 kHz) (fig. 2). Of 74 injection sites into to AH/POA region in 60 rats, ultrasonic vocalization was induced from 54 brain sites (73%). Sixteen injection sites fell outside the target area, with no vocalization elicited from these sites. No long calls were emitted after any of the pretreatments alone (saline, GABA, picrotoxin or bicuculline). Microinjection of CCh reliably induced ultrasonic vocalization in all accurately placed sites in the first set of injections ('a' groups) with an average peak frequency of 27 kHz (range: 19-38 kHz), an average call duration of 541.52 ms (range: 72-1328 ms), and an average bandwidth of .7 kHz (range: .3-1.5 kHz).

Some analyses were completed for the second set of injections (group 'b') in groups 1-6. This is discussed below in the individual groups as experimental conditions vary across 'b' groups.

<u>Experiment 1a: GABA (1 μg, 0.2 μl) pre-treatment</u>

The control condition consisted of a saline (0.2 μl) pre-treatment, followed by an injection of CCh (1.5 μg, 0.2 μl). Animals in the experimental condition received a pre-treatment of GABA (1 μg, 0.2 μl), followed by an equal volume of CCh (1.5 μg, 0.2 μl). CCh was injected five minutes following the pretreatment injection. Subsequent histological examination of injection sites revealed that cannula sites in two animals were outside of the target range (AH/POA). Of the remaining animals, all eight

vocalized in at least one condition. No significant difference was found between conditions for latency ($\underline{t}_{(7)}=1.812$, n.s.), response duration ($\underline{t}_{(7)}=2.054$, n.s.), or total number of calls ($\underline{t}_{(7)}=2.176$, n.s.) (Fig. 3-5).

Previous research has suggested that acoustic characteristics of ultrasonic vocalization in rats may convey important information to conspecifics. As a result acoustic parameters were measured by average call duration (ms), peak frequency (kHz) and average bandwidth (kHz) of calls after saline or GABA pre-treatment. As all responding animals vocalized in both conditions, analysis of acoustic parameters was completed for the eight vocalizing rats. No significant differences of acoustic characteristics were found for call duration, ($\underline{t}_{(7)}=1.593$, n.s.) (Fig. 6), or bandwidth ($\underline{t}_{(7)}=1.024$, n.s.) (Fig. 8). A significant difference was observed for peak frequency, ($\underline{t}_{(7)}=2.482$, p=.042) (Fig. 7), although this was not reproduced with other GABA doses.

Experiment 1b: Picrotoxin (0.2 µg, 0.2 µl) pre-treatment

Following the GABA treatment, animals in group one were placed in a GABA antagonist (picrotoxin) treatment group. In this second treatment condition, the effects of picrotoxin pre-treatment on CCh induced vocalization were examined. Animals were injected with a saline or picrotoxin pre-treatment in counterbalanced order. Five minutes later animals were injected with CCh (1.5 µg, 0.2 µl). All of the nine rats tested vocalized, with only three rats vocalizing only in both conditions (picrotoxin pretreatment, CCh treatment). No significant difference was found between conditions for latency ($\underline{t}_{(8)}=0.810$, n.s.), response duration ($\underline{t}_{(8)}=0.105$, n.s.), or total number of calls ($\underline{t}_{(8)}=0.247$, n.s.) (data not shown).

Picrotoxin did not significantly alter acoustic parameters as measured by average call duration, peak frequency, or bandwidth of calls. As only 3 animals responded in both conditions, these results are not included. It is difficult to interpret these results as meaningful with such a small sample size. In addition, interpretation of these results may be questionable as all rats had already received two prior injections and only 30% of animals consistently responded. As a result, the lack of vocalization may be attributable to damage from repeated injections or habituation to the testing environment. In the first case, animals may have ceased responding due to damage to the target site caused by the highly destructive nature of picrotoxin. This possibility is supported by the fact that when rats were injected again following the picrotoxin condition, repeating the GABA (ic) pretreatment, only one rat responded to CCh injection with vocalization and four rats responded with seizure behaviour. This is in sharp contrast to the original group 1 GABA condition where 8 rats vocalized and only one rat demonstrated seizure behaviour (data not presented here). Secondly, decreased vocalization may be the result of habituation to the testing environment. An animal's baseline anxiety level may decrease with repeated exposure to the recording chamber. If this is the case, it may be expected that 'alarm calls' could decrease with each additional exposure to the testing procedure.

Experiment 2a: GABA (2 μg, 0.2 μl) pre-treatment

As the pretreatment of group 1 with GABA (1 μg, 0.2 μl) did not produce significant differences in vocalization parameters, the dose of GABA was increased to 2 μg, 0.2 μl for the second group. CCh was administered five minutes after the pretreatment. Previous research has indicated that effectiveness of a GABA agonist on altering vocalization response was dose dependent (Hawkins, 1999). In addition, it is expected that the concentration of a substance that may be required to obtain a quantifiable response may exceed the entire endogenous quantity within a given structure (Myers, 1974). An increase of 1 μg was administered in an attempt to determine whether results were due to inadequate dose of GABA, or a lack of involvement of the GABAergic system in the production of ultrasonic vocalization in rats. Rats were once again treated in counterbalanced order, with all rats receiving both conditions (saline and GABA pretreatment). All ten rats vocalized in at least one treatment condition. Histological examination revealed that cannulae were accurately placed within the AH/POA in all rats. Local pretreatment with GABA did not significantly alter response latency ($\underline{t}_{(9)}$=.020, n.s.), response duration ($\underline{t}_{(9)}$=.319, n.s.), or number of calls ($\underline{t}_{(9)}$=.084, n.s.) (Fig. 3-5).

Six of a total of ten rats vocalized in both conditions, and were used in analysis of acoustic parameters. GABA (2 μg, 0.2 μl) pre-treatment did not alter acoustic characteristics as measured by average call duration ($\underline{t}_{(5)}$=1.189, n.s.), peak frequency ($\underline{t}_{(5)}$=1.513, n.s.) bandwidth ($\underline{t}_{(5)}$=1.781, n.s.) (Fig. 6-8).

Experiment 2b: GABA (5 µg, 0.2 µl) pre-treatment

As no effects were found in group 2 with the initial injection of 2 µg, 0.2 µl, the GABA dose was increased to 5 µg, 0.2 µl. All other treatment conditions remained the same. Seven of a total of eight rats vocalized in at least one condition. Pretreatment with this increased dose of GABA (5 µg, 0.2 µl) did not significantly alter vocalization characteristics as measured by latency ($t_{(6)}$=.581, n.s.); response duration ($t_{(6)}$=.756, n.s.); number of calls ($t_{(6)}$=.646, n.s.).

Only three animals vocalized in both treatment conditions, restricting analysis of acoustic parameters to *df*=2. No significant differences were observed for acoustic parameters as measured by average call duration ($t_{(2)}$= .102, n.s.), peak frequency($t_{(2)}$= .350, n.s.) and bandwidth ($t_{(2)}$=.443, n.s.). These results represent data from only 3 rats as not all rats vocalized in both conditions. This data is presented as further evidence for the lack of influence of GABA at increasing dosages. Interpretation of these results is limited as rats in all conditions decreased vocalizing with multiple injections. As a result data not representing the first experiment with each group is not presented graphically.

To determine if vocalization characteristics differed significantly with successive injections, paired-samples *t*-tests were completed comparing the control conditions in group 2a versus group 2b, and comparing the experimental conditions in group 2a versus 2b. In the control condition, a total of seven rats vocalized. Analysis between group 2a and 2b did not reveal any significant differences in vocalization characteristics as measured by latency ($t_{(6)}$=.344, n.s.); response duration ($t_{(6)}$=.416, n.s.); or number of calls ($t_{(6)}$=.517, n.s.). Similarly, no differences in acoustic

characteristics were found for the GABA pretreatment group despite the increased dosage. Eight animals vocalized in the experimental condition, and were included in the analysis of vocalization characteristics as measured by latency ($t_{(7)}=1.144$, n.s.); response duration ($t_{(7)}=-1.318$, n.s.) and number of calls ($t_{(7)}=.533$, n.s.).

Only three animals responded in both control conditions in group 2a and 2b. Similarly, vocalizing animals was limited to three in the experimental condition. As a result, analyses were not completed for acoustic parameters as measured by average call duration, peak frequency, and bandwidth.

Experiment 3a: Bicuculline (0.3 µg, 0.1 µl)

Histological verification revealed accurate cannulae implantation within the AH/POA in eight animals. All of these animals vocalized in at least one of the conditions. Although Group 1 animals received a GABA antagonist pretreatment (picrotoxin), this was administered following the initial treatment with GABA. Hence, rats had already received two treatments into the injection site. In addition, previous research has found bicuculline (Fernandez-Guasti, Larsson, & Vega-Sanabria, 1986) and not picrotoxin (De Vry, Benz, Schreiber, & Traber, 1993) effective in altering UV. In an attempt to verify the non-responsiveness of animals, Group 3 animals were pretreated with bicuculline, a powerful GABA antagonist and saline in counterbalanced order. Previous research has found that pretreatment with bicuculline has significantly altered 22kHz vocalization associated with sexual behaviour in rats (Fernandez-Guasti, Larsson, & Vega-Sanabria, 1986). Pretreatment with a similar dose of bicuculline to that in the Fernandez-Guasti et al. (1986) study did not affect carbachol induced 22kHz

vocalization in any of the measures: latency: ($\underline{t}_{(7)}$=1.015, n.s.); response duration: ($\underline{t}_{(7)}$=.327, n.s.); number of calls: ($\underline{t}_{(7)}$=.277, n.s.) (Fig. 3-5).

All eight animals vocalized in both conditions. As a result analysis was completed for eight animals. Acoustic characteristics as measured by average call duration ($\underline{t}_{(7)}$=.145, n.s.), peak frequency ($\underline{t}_{(7)}$= 1.070, n.s.) and bandwidth ($\underline{t}_{(7)}$=.311, n.s.) did not differ significantly in bicuculline pre-treated versus control rats (Fig. 6-8).

Experiment 3b: Bicuculline (0.3 µg, 0.1 µl)

As stated earlier, results can only be attributed to local drug action if they are acting at the site of injection. The time period between pretreatment and CCh injection was decreased from five minutes to two minutes to account for the possibility that bicuculline may diffuse quickly from the target site or be metabolized prior to CCh injection. Five of a total of eight responding animals vocalized in both conditions. Results were consistent with the original bicuculline pretreatment condition with no significant differences in acoustic parameters: latency ($\underline{t}_{(4)}$=.637, n.s.); response duration ($\underline{t}_{(4)}$=.918, n.s.); number of calls ($\underline{t}_{(4)}$=.216, n.s.). Analyses was not completed for acoustic parameters as only two rats vocalized in both conditions restricting analysis to *df*=1.

It is noteworthy that only five rats vocalized in this condition, less than the eight rats responding in the original group 3 condition. Again, this may be the result of damage to nerve tissue from multiple injections. Nevertheless, bicuculline pretreatment did not significantly alter vocalization in either condition (2 minute versus 5 minute delay period between pretreatment and CCh injection).

Experiment 4a: GABA (20 μg, 0.2 μl)

As a result of continued lack of evidence of GABA influence upon the vocal response at the low dose level administered, the GABA dose was arbitrarily increased to 20 μg, 0.2 μl. This increase was to determine if lack of influence was related to ineffective dosage of GABA. CCh was administered 2 minutes following pretreatment. Histological verification revealed accurate cannulae placement in nine out of ten rats. All nine rats were included in analyses of vocalization characteristics. Consistent with lower dosages, no significant effects were observed for latency ($t_{(8)}$=.202, n.s.); response duration: ($t_{(8)}$=.551, n.s.); number of calls: ($t_{(8)}$=.536, n.s.) (Fig. 3-5).

Analysis of acoustic parameters included the six animals that vocalized in both the control and experimental condition. Increased GABA dosage administered at 20 μg, 0.2 μl did not significantly alter acoustic parameters as measured by average call duration ($t_{(5)}$=.105, n.s.), peak frequency ($t_{(5)}$= .144, n.s.), or average bandwidth ($t_{(5)}$=.456, n.s.) (Fig. 6-8).

Experiment 4b: GABA (20 μg, 0.2 μl)

To confirm the results found in group 4a, rats in group 4 were retested using the identical dose of GABA (20 μg, 0.2 μl). All other experimental conditions remained unchanged. Seven of a total 9 rats vocalized in this treatment condition (in contrast with the original 9 vocalizing rats in group 4a). Consistent with results found in group 4a, no significant effects were observed for latency ($t_{(6)}$=.847, n.s.); response duration: ($t_{(6)}$=.294, n.s.); number of calls: ($t_{(6)}$=1.481, n.s.). As only 4 animals vocalized in both

conditions, acoustic characteristics are derived from a reduced number of rats: average call duration ($t_{(3)}$=1.110, n.s.), peak frequency ($t_{(3)}$= .955, n.s.), or average bandwidth ($t_{(3)}$=1.199, n.s.).

Further analyses were completed comparing group means for group 4a and 4b in both the control and experimental conditions. As all other experimental procedures were identical, any significant differences in vocalization characteristics would be attributable to deterioration of response with multiple injections or the result of an order effect (i.e., group 4a was not counterbalanced with group 4b). Comparison of group 4a and 4b did not reveal any significant differences in vocalization characteristics as measured by latency ($t_{(8)}$=1.233, n.s.); response duration ($t_{(8)}$=.600, n.s.) and number of calls ($t_{(8)}$=.183, n.s.). Similarly, no differences in acoustic characteristics were found for the GABA pretreatment group: latency ($t_{(8)}$=.425, n.s.); response duration ($t_{(8)}$=.363, n.s.) and number of calls ($t_{(8)}$=1.643, n.s.).

As only three animals vocalized in both 'a' conditions and 'b' conditions further analyses were not completed on acoustic parameters as measured by average call duration, peak frequency or bandwidth.

Experiment 5a: GABA (40 μg, 0.2 μl)

To confirm earlier results and to look for possible trends, Group 5 animals received a pretreatment of 40μg, 0.2 μl of GABA. The CCh dose was reduced to 1μg, 0.2 μl, still effective in eliciting vocalization and more likely to be influenced by GABA. Animals were injected unilaterally in the left cannula. Histological verification revealed accurate cannulae placement in seven out of ten animals and this data was

included for analysis. Despite this high dose, GABA did not influence the vocal response as measured by latency: ($\underline{t}_{(9)}$=.853, n.s.); response duration: ($\underline{t}_{(9)}$=.103, n.s.); number of calls: ($\underline{t}_{(9)}$=.803, n.s.) (Fig. 3-5).

Analyses of acoustic characteristics as measured by average call duration, frequency, and bandwidth (Fig. 6-8) were not appropriate due to the small number of animals vocalizing in both conditions (N=2)

Experiment 5b: GABA (40 µg, 0.2 µl)

Group 5 animals were injected a second time with a pre-treatment of 40µg, 0.2 µl of GABA in counterbalanced order with saline, but in the right cannula. This was to confirm that this increased dose of GABA did not influence acoustic characteristics. Using the right cannula allowed for a fresh site. Unlike earlier repeated drug administrations, a decrease in the number of rats vocalizing could not be attributed to multiple injections in the same target area (assuming correct cannula placement). Histological verification revealed that only three right cannulae were accurately placed within the AH/POA (see Fig. 9). As a result, further analyses were not appropriate (N=3). Similarly, vocalization characteristics were not compared between group 5a and 5b due to the small number of rats vocalizing in group 5b.

Experiment 6a: GABA (40 µg, 0.2 µl)

Animals in Group 6 received the identical experimental pretreatment administered to those in Group 5; GABA 40µg, 0.2 µl. Both pretreatments were followed two minutes later with CCh (1µg, 0.2 µl). One alteration was made to the drug

administration. Animals in Group 6 were habituated to the recording chamber prior to participation in experimentation. Rats were placed into the recording chamber for ten minutes on three separate occasions, three days apart. This was to ensure that a lack of familiarity with the cage was not contributing to a heightened anxiety that may lead to greater likelihood of vocalization with initial placement in the recording chamber. Previous groups (1-5) were handled on three occasions prior to experimentation, but were not actually placed in the recording chamber (except for after sham injections).

Vocalization parameters for Group 5 and 6 were then compared using a two-factor ANOVA to ensure there were no differences between groups receiving handling versus the group receiving cage habituation. The two-factor analysis of variance showed a significant main effect for group, $F_{(1,15)}=5.664$, $p=.029$; animals in group 5a had a significantly longer latency than those in 6a (this is in contrast to what would be predicted if prior exposure to the testing environment reduced UV). There was no significant main effect for latency condition (control versus experimental), $F_{(1,15)}=1.167$, n.s. The interaction between group and latency condition was not significant $F_{(1,15)}=.284$, n.s.

Analyses of response duration condition (control versus experimental) and group (5a and 6a) showed no significant main effect for group, $F_{(1,15)}=3.770$, n.s. There was no significant main effect for response duration condition, $F_{(1,15)}=.038$, n.s. In addition, no significant interaction emerged between group and response duration condition, $F_{(1,15)}=.000$, n.s.

Similar analyses with group (5a and 6a) and total calls condition (control versus experimental) revealed no significant main effect for the group factor, $F_{(1,15)}=3.463$, n.s.

and no significant effect for total calls condition factor, $\underline{F}_{(1,15)}$=.844, n.s.; and no significant interaction between group and call condition, $\underline{F}_{(1,15)}$=.123, n.s.

In addition, a lack of significant effects in Group 6 confirms that pretreatment of GABA even at high doses 40μg, 0.2 μl, does not influence vocalization response as measured by latency ($\underline{t}_{(9)}$=.843, n.s.), response duration ($\underline{t}_{(9)}$=.318, n.s.) and number of calls ($\underline{t}_{(9)}$=.489, n.s.). All of the ten animals vocalized in group 6a, with nine of ten rats vocalizing in both conditions. Similar results were observed for acoustic parameters as measured by average call duration ($\underline{t}_{(8)}$=.205, n.s.), peak frequency ($\underline{t}_{(8)}$=.169, n.s.) bandwidth ($\underline{t}_{(8)}$=.472, n.s.) (Fig. 6-8).

Experiment 6b: GABA (40 μg, 0.2 μl)

Animals in group 6b received identical treatment to those in 6a with an experimental pretreatment of GABA 40μg, 0.2 μl. All animals were injected in the left cannula. As all animals in group 6b were habituated to the vocalization chamber, any reduction in the number of rats vocalizing would not likely be from habituation. Consistent with findings in earlier groups, fewer rats vocalized in the second experiment (6 out of 9 total). GABA pretreatment did not alter vocalization as measured by latency ($\underline{t}_{(5)}$=1.207, n.s.), response duration ($\underline{t}_{(5)}$=-1.880, n.s.), and number of calls ($\underline{t}_{(5)}$=-1.736, n.s.).

Five of the six rats vocalized in both pretreatment conditions and were included in the analysis of acoustic parameters and no significant differences were found among groups as measured by peak call duration ($\underline{t}_{(4)}$=-.594, n.s.), frequency ($\underline{t}_{(4)}$=.441, n.s.) bandwidth ($\underline{t}_{(4)}$=.181, n.s.).

A paired samples *t*-test between group 6a and 6b did not reveal any significant differences in vocalization characteristics as measured by latency ($\underline{t}_{(5)}=1.457$, n.s.); response duration ($\underline{t}_{(5)}=.965$, n.s.); or total number of calls $\underline{t}_{(5)}=1.629$, n.s. for the saline pretreatment group.

Similarly, no differences in acoustic characteristics were found for the GABA pretreatment group despite the increased dosage: latency ($\underline{t}_{(5)}=1.432$, n.s.); response duration ($\underline{t}_{(5)}=.062$, n.s.) and number of calls ($\underline{t}_{(5)}=.748$, n.s.).

Group 5 and 6: GABA (40 µg, 0.2 µl)

To increase power and the likelihood of finding meaningful results groups 5 and 6 were combined for statistical analysis. Both groups were subjected to identical treatment conditions, except that group 6 was habituated to the vocalization recording chamber. No significant differences were found between the groups, with the exception of call latency. This difference is likely the result of individual variations and not treatment conditions (as discussed earlier). Significant differences in vocalization characteristics did not emerge with the combination of groups 5 and 6: latency ($\underline{t}_{(16)}=-1.101$, n.s.), response duration ($\underline{t}_{(16)}=.201$, n.s.), number of calls ($\underline{t}_{(16)}=-.962$, n.s.), average call duration ($\underline{t}_{(10)}=.715$, n.s.), frequency ($\underline{t}_{(10)}=.437$, n.s.) bandwidth ($\underline{t}_{(10)}=.800$, n.s.) (data not shown).

Histological Verification of Injection Sites

Localization of the mapping sites is illustrated in Fig. 9. Only those sites which received injections are shown. Sites effective in eliciting 22 kHz vocalization are marked by a filled circle. Injection sites not effective in eliciting 22kHz vocalization are marked with an unfilled circle. Distribution of filled circles illustrate that vocalization was consistently elicited from microinjection successfully administered to the target area (AH/POA). Unsuccessful sites typically fell outside the target area.

Summary of Results

Local pretreatment with GABA (1-40 µg, 0.2 µl) or GABA antagonists (picrotoxin or bicuculline) did not significantly affect CCh induced ultrasonic vocalization in any of the groups tested. The one exception was peak frequency in group 1. Each group received sham injections at the beginning and end of the testing sequence to ensure that vocalization was not the induced by the handling experience alone. None of the rats vocalized during or following the sham injection. As GABA pretreatment did not significantly alter total number of calls, analysis was completed for number of calls for the first and second minute of vocalization. This was done to ensure that, if pretreatment effects were not long lasting, they were not overlooked by totaling ten minutes of calls. No significant differences were found for total number of calls in the first and second minute of the response (data not shown).

Discussion

Pretreatment with GABA, within the dose range 1-40 μg, or a GABA antagonist (picrotoxin or bicuculline) had marginal or no effect on the number of CCh-induced calls (Fig. 5), response latency (Fig.3) and response duration (Fig. 4). In addition, pretreatment with GABA and its antagonists did not affect acoustic parameters as measured by average call duration (Fig. 6), peak frequency (Fig. 7) or average bandwidth (Fig. 8) at any of the doses used. The results suggest that cholinoceptive neurons of the region of the medial vocalization strip are not under direct GABAergic control.

Although previous research has found that injection of a GABA antagonist into the AH/POA reduces ultrasonic vocalization as measured by response duration (Fernández-Guasti et al., 1985), we were not able to replicate this result. As these results were found on 22 kHz calls associated with sexual behaviour in rats, it may be speculated that different mechanisms underlie UV production in different behavioural situations. It is well established that ultrasonic 'alarm calls' differ in their characteristics (rate, frequency. length, sound pressure level, pattern) depending on the situation (Miczek et al., 1991; Sales, 1972; Sales & Pye, 1974). One possibility is that calls serving different behavioural functions may have slightly different mechanisms involved in their production. However, this explanation does not account for findings that local GABA microinjections into the SN increase tail pinch invoked UV response (Hawkins et al., 1999). The direct local injection of drugs to the AH/POA was done to control for widespread inhibitory effects of GABA throughout the central nervous systems.

Further evidence for the role of GABA in the control of emission of ultrasonic vocalization comes from research finding suppression of footshock-induced calls in adult rats when injected with diazepam interperitoneally (ip) (Cuomo, Cagiano, De Salvia, Maselli, Renna, & Racagni, 1988; De Vry, Benz, Schreiber, & Traber, 1993; Tonoue et al., 1987) The role of the benzodiazepine-GABA receptor-chloride channel complex in reduction of ultrasonic calls comes also from studies of isolation calls in rat pups (Insel et al., 1986). Behavioural research has supported these findings, with the observation of a significantly reduced response by peripheral pretreatment with benzodiazepines (Beckett, Aspley, Graham, & Marsden, 1996). However, the findings have been inconsistent in that pretreatment with benzodiazepines has not influenced tail shock induced UV (Molewijk, Van Der Poel, Mos, Van Der Heyden & Olivier, 1995; Vander Poel, Noach, Miczek, 1989).

In addition to their clinical potency, benzodiazepines are of interest because their anxiolytic effects appear to be mediated by a specific, membrane-bound receptor (functionally linked to the GABA-A receptor and a chloride ion channel) with a well-defined neuroanatomic distribution (Insel, Hill, & Mayor, 1986). Although diazepam and other benzodiazepine derivatives have exerted their effects after ip injection, this does not appear to be the result of sedative or muscle-relaxant properties. Administration of desipramine, an atypical antidepressant (Cuomo et al., 1988) or haloperidol, a dopamine receptor antagonist (De Vry et al., 1993) at dose levels inducing sedation does not affect UV. In addition, Molewijk et al. (1995) found no direct effect between reduction of motor activity and reduction of UV. Another

possibility is that changes in ultrasonic vocalization are secondary to other behavioural changes.

Inconsistencies in results might also be explained by neurotransmitter interactions or mutual interdependence of neuronal populations (Dilsaver, 1986). Neuro-pharmacological effects of drugs may not be revealed by reductionist accounts of their influences on a given neurotransmitter system studied in isolation. Interaction and interregulation between neurotransmitter systems is considered basic to normal neural functioning. In addition to GABAergic evidence, other neurotransmitter systems have been implicated in the production of ultrasonic vocalizations. Suppression of ultrasonic vocalization has been achieved with seratonin receptor agonists (De Vry et al., 1993; Mos et al., 1991; Sánchez & Mørk, 1999), N-methyl-D-aspartate glutamate (NMDA) receptor agonists (De Vry et al., 1993), adrenergic agonists (Mos et al., 1991), selective dopamine (D2) receptor agonists, opiates (Calvino et al., 1996; Panksepp et al., 1988) and analgesics (Calvino et al., 1996).

It has been suggested that 22 kHz vocalization in aversive situations presents a model of anxiety (Cuomo et al., 1988). As stated earlier benzodiazepines have been inconsistent in their effects on alarm calls. In addition, tricyclic and tetracyclic, as well as some atypical antidepressants and a monamineoxidase inhibitor showed no reducing effects, or reduced vocalization only at high doses. The cholecystokinin-B (CCK-B) receptor is thought to be involved in the development of anxiety. However, previous research has found minimal influence of drugs acting at the CCK-B receptor on ultrasonic vocalization (Voits, Beckett, Marsden, & Fink, 1999). Consistent with the results of the current study, picrotoxin has shown either no, or very weak effects on

ultrasonic vocalization (De Vry et al., 1993). As a result it has been suggested that this ultrasonic vocalization model specifically measures anxiolytic effects. Ultrasonic vocalizations by adult rats that are exposed to painful and startle stimuli are attenuated by benzodiazepines. As stated previously, low, non-sedative doses of diazepam decreased ultrasounds in rats presented with electric footshock (Cuomo et al., 1988; Tonoue et al., 1987), but not with tail shock (Van der Poel et al., 1989). In humans, diazepam is very effective in the treatment of general anticipatory anxiety, but considerably less so in panic attacks (Rickels & Schweitzer, 1987). It has been suggested that the sensitivity of conditioned ultrasonic vocalization to seratonin uptake inhibitors versus the insensitivity to classical benzodiazepines closely resembles the psychopharmacology of panic disorder whereby benzodiazepines are less effective in the treatment of panic attacks than generalized anxiety disorder (Molewijk et al., 1995). In addition, a ‘dual fear hypothesis’ has been suggested, involving two pathways mediating learned anxiety and unconditioned anxiety/fight-flight reactions (Sanchez & Mørk, 1999). Further pharmacological exploration of the mechanisms for different ultrasonic vocalizations may reveal these sounds represent the evolutionary precursors to human expressions of anxiety (Miczek et al., 1991).

The results of the current study suggest that GABA has no direct role in the production of ultrasonic control in cholinoceptive neurons of the medial vocalization strip. However, the role of GABA in the production of ultrasonic vocalization cannot be judged on the basis of the current data. It has been suggested that intraanimal variability in acoustic parameters of the vocalization response (as measured by latency and response duration) could reduce the sensitivity of pharmacological testing (Jourdan,

Ardid, Chapuy, Le Bars, & Eschalier, 1997). Therefore, it may be necessary to increase the number of animals studied to observe drug effects. In addition, numerous studies examining the effects of GABA and benzodiazepine derivatives employed procedural differences that may account for inconsistent results (e.g., injections were given bilaterally or ip versus i.c., injecting cannula kept in place for additional minute after injection to allow for diffusion) (Hawkins et al., 1999). Finally, injections of GABA influenced only a limited portion of the medial cholinoceptive strip, which could be insufficient in antagonizing or modifying the response. Simultaneous injections of GABA into several cannulae along the strip could be effective.

In summary, the current results suggest that cholinoceptive neurons of the medial vocalization strip are not under direct GABAergic control. However, the role of GABA in the production of UV cannot be determined from the current results. Numerous studies have found that peripheral pretreatment with benzodiazepines has altered the vocalization response. In addition, central and peripheral application of GABA agonists and antagonists have influenced UV in a predictable manner. It is likely that a number of factors combine to create inconsistencies in various studies including varying eliciting stimuli, intraanimal variability, incomparable experimental procedures and complex neurotransmitter interplay. It is also conceivable that otherwise similar ultrasonic calls emitted in substantially different biological situations may have different neural/neurochemical mechanisms responsible for the production of these calls. Further pharmacological exploration is required to elucidate the underlying mechanisms involved in the production of alarm calls in rats.

Future Research

Using animal models is useful in understanding the neural substrates underlying human behaviour to gain an understanding of neurotransmitters involved and potential drug treatments (Bourin, 1997). Ethopharmacology applies the methods and concepts of ethology to the study of drug-induced changes in behaviour (Mos et al., 1991). This leads to new insights on how psychoactive drugs modify behaviour and what molecular mechanisms underlie these actions. Also, studies using different drugs may shed light on the organization and functional significance of various behavioural elements. This knowledge may be further used for the development of specific psychoactive drugs for therapeutic purposes. There is evidence for a medial cholinoceptive strip in the production of 'alarm calls' in rats. In addition, there is evidence for a cholinergic mechanism in the pathophysiology of affective disorders in humans (Dilsaver, 1986). Acetylcholine activates the limbic-hypothalamic-pituitary-adrenal axis (LHPA) under stressful conditions. Regulation of the LHPA axis involves interactions among several neurotransmitter systems. Noerepinephrine, endogenous opiods, and GABA tend to inhibit the axis. There is strong support for the hypothesis that cholinergic mechanisms are operative in the pathophysiology of depressive disorders. If the nature of cholinergic systems is to be properly understood, interaction with other neurotransmitter systems must be carefully understood and clarified.

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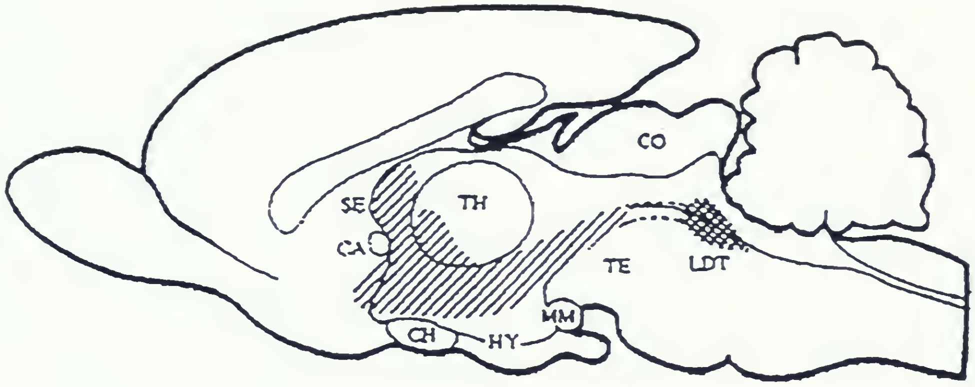

Figure 1. Midsagittal section through the rat brain with the cholinoceptive strip of medial structures (hatched area) from which the local application of carbachol induced behavioural response with the 22 kHz type of alarm calls as its main manifestation. The strip includes rostral reticular formation, prerubral field, zona incerta, dorsal hypothalamus, para- and pariventricular hypothalamic nuclei, medial hypothalamic area, anterior hypothalamic-preoptic area, diagonal band of Broca, medial-ventral pallidum, anteromedial nucleus accumbens, and septum. This diagram and explanation have been reproduced with permission from S. Brudzynski, 1998. The cholinergic innervation originates from the laterodorsal tegmental nucleus (LDT) (cross hatched area). Abbreviations: CA – commissura anterior, CH – optic chiasm, CO – colliculi, HY – hypothalamus, LDT – laterodorsal tegmental nucleus, MM – mammillary bodies, SE – septum, TH – thalamus, TE – tegmentum.

Sonagraphic analysis of a typical 22 kHz alarm call

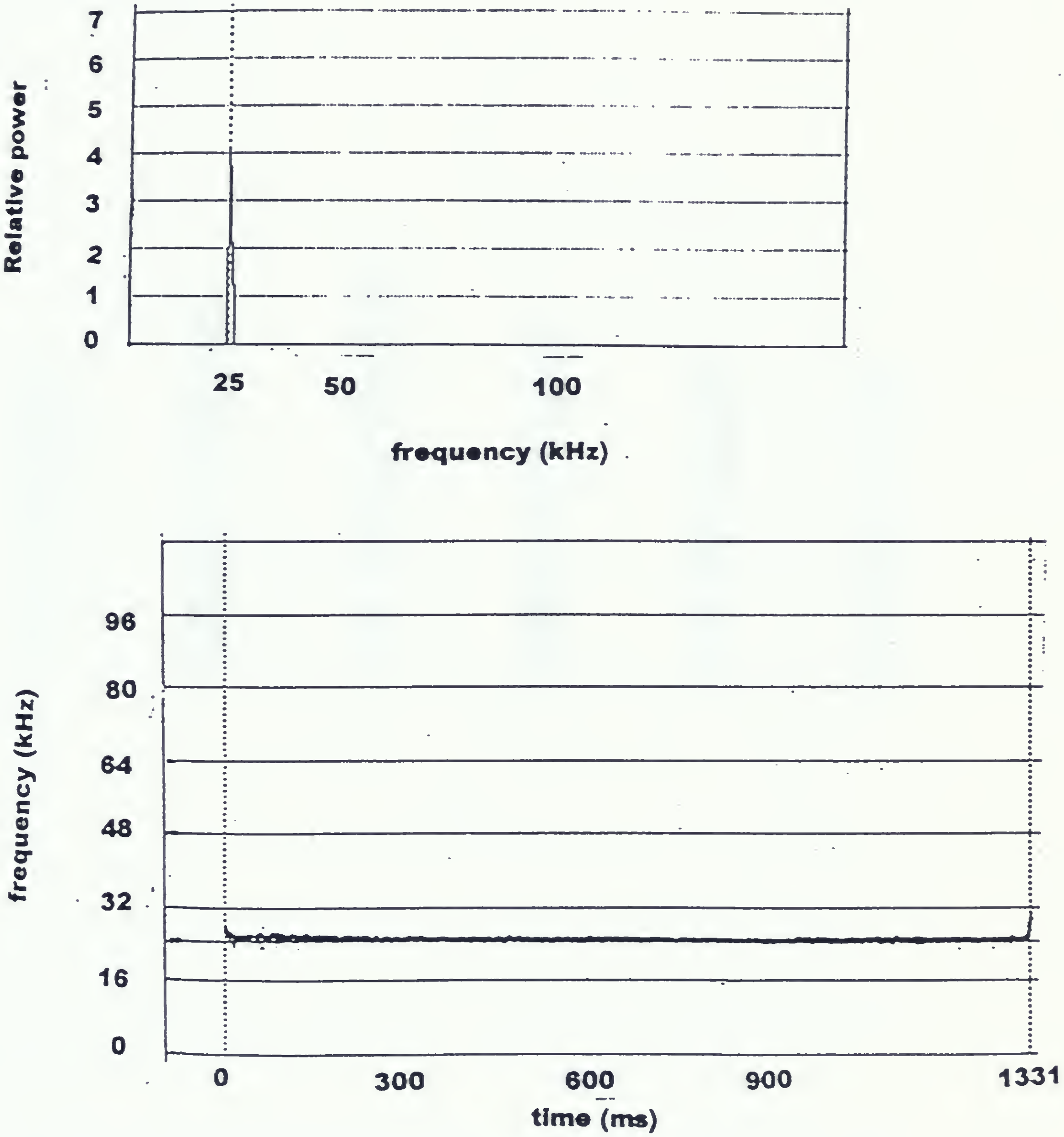

Figure 2. Sonagraphic analysis of acoustic parameters of a typical 22 kHz alarm call. *Upper diagram*: power spectrum of a single call shown on a sonagram in the *lower diagram*. The call has the following parameters: call duration=1331 ms, average sound frequency=25 kHz, average bandwidth=4.5 kHz. The vertical dotted lines represent manually set computer cursors.

Effects of drug pre-treatment on CCh induced 22kHz alarm calls

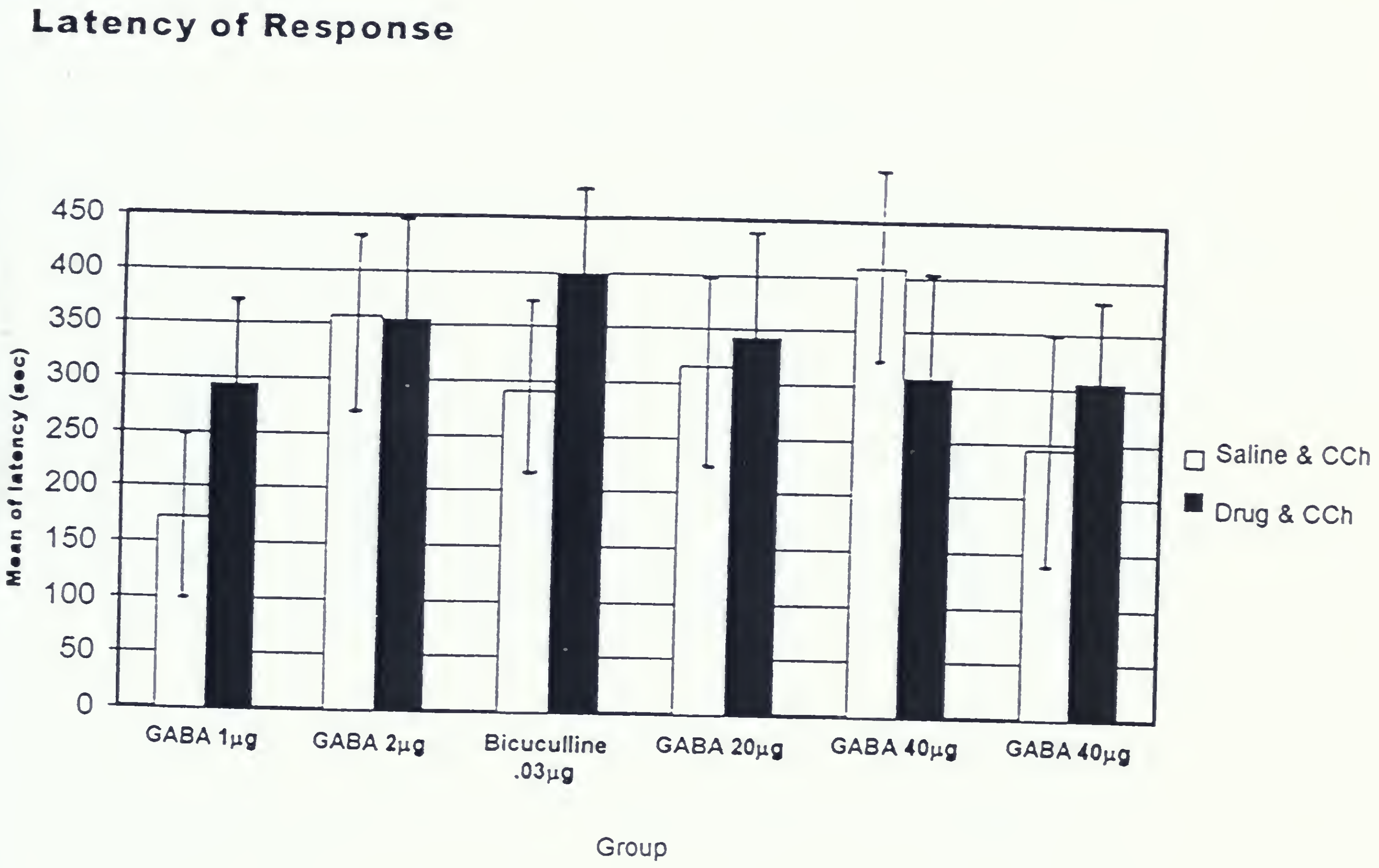

Figure 3. The effect of drug pre-treatment (GABA 1-40µg ic and bicuculline 0.03µg ic) on latency of CCh-induced vocalization. Each bar represents a mean of the latency of response in seconds. Each pre-treatment was administered 2-5 minutes prior to injection of CCh (1.5µg in 0.2µl saline). There was no significant difference in average latency between the control vehicle (blank bars) and the drug injections (filled bars). Vertical lines represents SEMs. Statistical results are as follows:

Group1 (GABA 1 µg): ($t_{(9)}$=1.812, n.s.), group 2 (GABA 2 µg): ($t_{(9)}$=-0.20, n.s.), group 3 (bicuculline): ($t_{(9)}$=1.015, n.s.), group 4 (GABA 20 µg): ($t_{(9)}$=0.202, n.s.), group 5 (GABA 40 µg): ($t_{(9)}$=.853, n.s.), group 6 (GABA 40 µg): ($t_{(9)}$=-.843, n.s.)

N=10 for all groups except group 4, n=9.

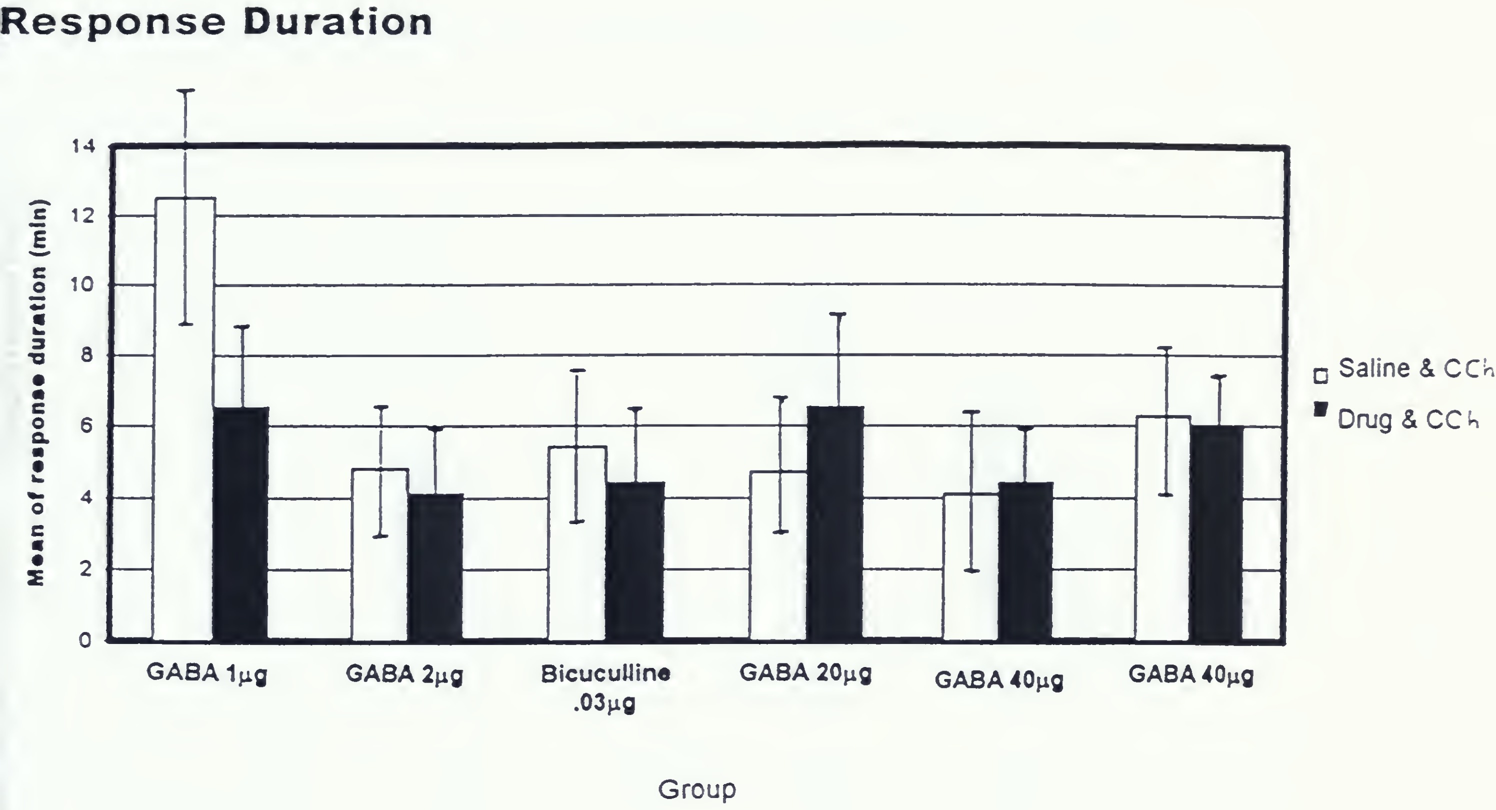

Figure 4. The effect of drug pre-treatment (GABA 1-40µg ic and bicuculline 0.03µg ic) on total duration of CCh-induced vocalization. Each bar represents a mean of the summed response duration in minutes. Each pre-treatment was administered 2-5 minutes prior to injection of CCh (1.5µg in 0.2µl saline). There was no significant difference in response duration between the control vehicle (blank bars) and the drug injections (filled bars). Vertical lines represent SEMs. Statistical results are as follows:

Group1 (GABA 1µg): ($t_{(9)}$=-2,054, n.s.), group 2 (GABA 2µg): ($t_{(9)}$=-.319, n.s.), group 3: (bicuculline) ($t_{(9)}$=-.327, n.s.), group 4 (GABA 20 µg): ($t_{(8)}$=.551, n.s.), group 5 (GABA 40 µg): ($t_{(9)}$=.103, n.s.), group 6: ($t_{(9)}$=.318, n.s.)

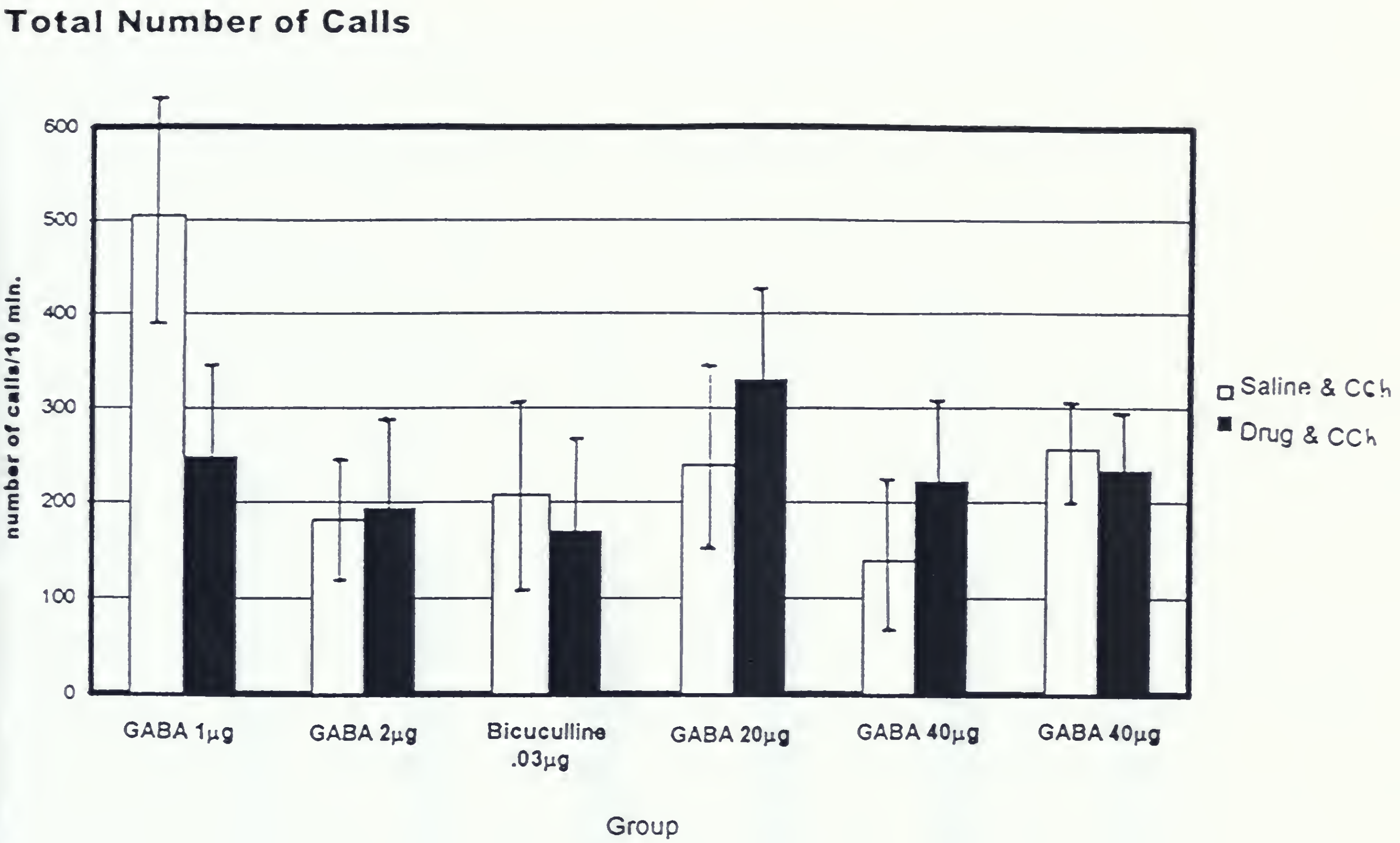

Figure 5. The effect of drug pre-treatment (GABA 1-40μg ic and bicuculline 0.03μg ic) on total number of calls induced by CCh. Each bar represents a mean of the total number of calls/10 min. Each pre-treatment was administered 2-5 minutes prior to injection of CCh (1.5μg in 0.2μl saline). There was no significant difference in total number of calls between the control vehicle (blank bars) and the drug injections (filled bars). Vertical lines represent SEMs. Statistical results are as follows:

Group1 (GABA 1 μg): ($t_{(9)}$=2.176, n.s.), group 2 (GABA 2 μg): ($t_{(9)}$=-.084, n.s.), group 3 (bicuculline): ($t_{(9)}$=.277, n.s.), group 4 (GABA 20 μg): ($t_{(8)}$=-.536, n.s.), group 5 (GABA 40 μg): ($t_{(9)}$=-.803, n.s.), group 6 (GABA 40 μg): ($t_{(9)}$=-.489)

N=10 for all groups except group 4, n=9.

Effects of drug pre-treatment on acoustic parameters of CCh induced 22kHz alarm calls

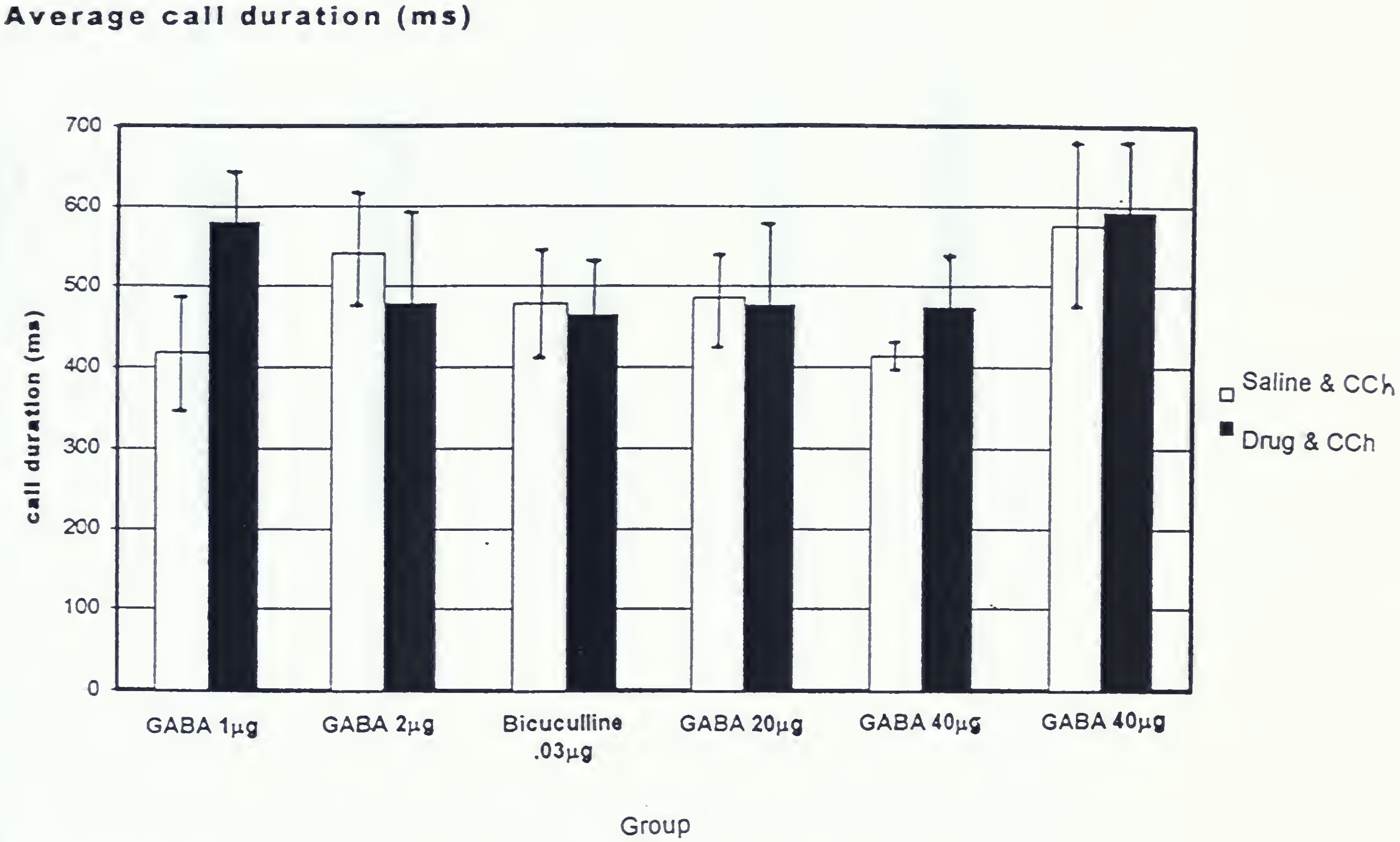

Figure 6. The effect of drug pre-treatment (GABA 1-40μg ic and bicuculline 0.03μg ic) on average duration of single calls induced by CCh. Each bar represents average call duration in ms. Each pre-treatment was administered 2-5 minutes prior to injection of CCh (1.5μg in 0.2μl saline). There was no significant difference in call duration between the control vehicle (blank bars) and the drug injections (filled bars). Vertical lines represent SEMs. Statistical results are as follows:

Group1 (GABA 1 μg): ($t_{(7)}$=-1.593, n.s.), group 2 (GABA 2 μg): ($t_{(5)}$=1.189, n.s.), group 3 (bicuculline): ($t_{(7)}$=.145, n.s.), group 4 (GABA 20 μg): ($t_{(5)}$=.105, n.s.).,group 5 (40 μg): ($t_{(1)}$=-.231, n.s.), group 6 (40 μg): ($t_{(8)}$=-.205, n.s.)

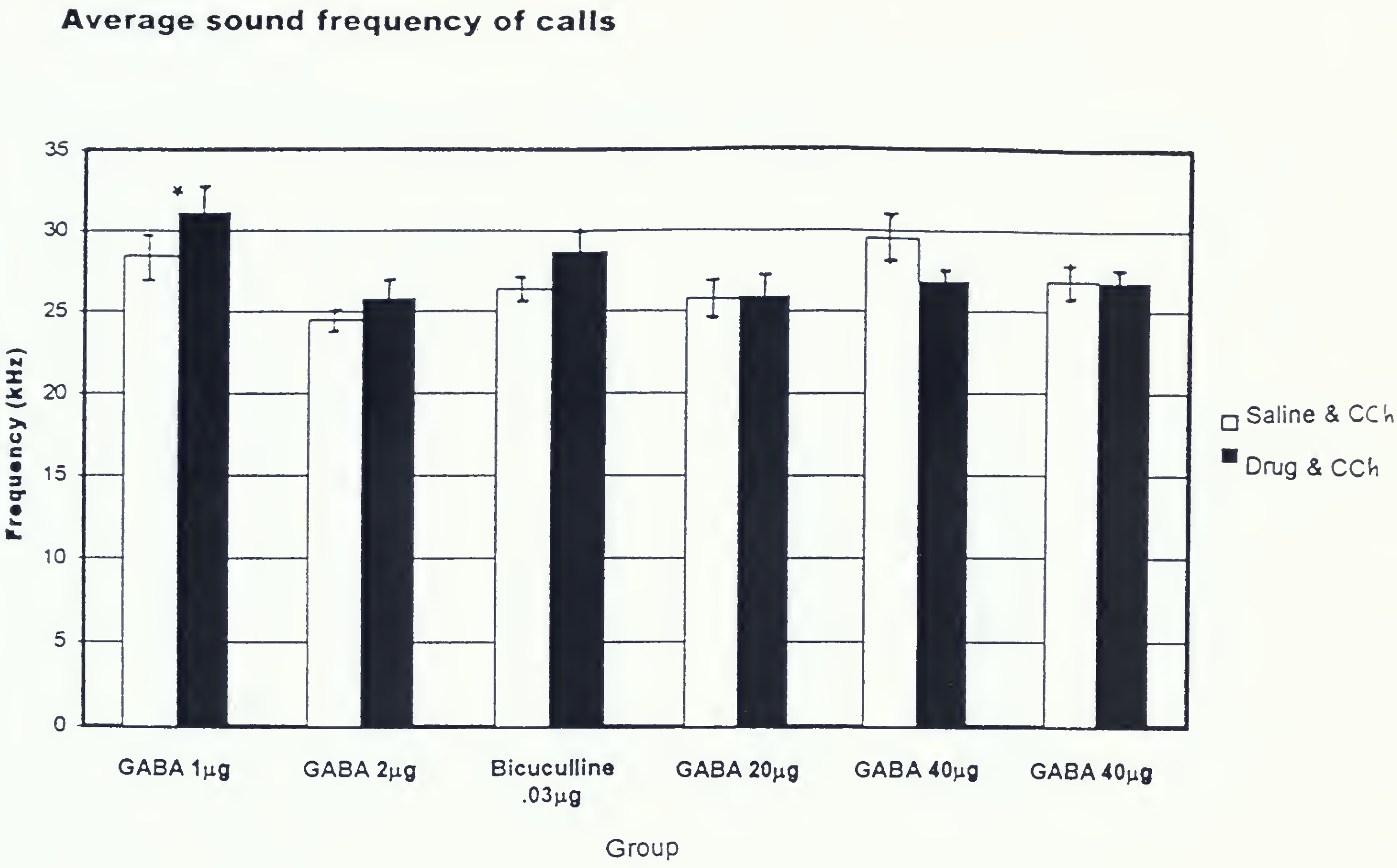

Figure 7. The effect of drug pre-treatment (GABA 1-40µg ic and bicuculline 0.03µg ic) on average peak frequency of CCh-induced calls. Each bar represents average peak frequency per treatment condition in kHz. Each pre-treatment was administered 2-5 minutes prior to injection of CCh (1.5µg in 0.2µl saline). There was no significant difference in average sound frequency between the control vehicle (blank bars) and the drug injections (filled bars), with the exception of Group 1 (asterisk). Vertical lines represent SEMs. Statistical results are as follows.

Group1 (GABA 1 µg): ($t_{(7)}$=-2.482, p=.042), group 2 (GABA 2 µg): ($t_{(5)}$=-1.513, n.s.), group 3 (bicuculline): ($t_{(7)}$=-1.070, n.s.), group 4 (GABA 20 µg): ($t_{(5)}$=-.144, n.s.)., group 5 (GABA 40 µg): ($t_{(1)}$=1.013, n.s.), group 6 (GABA µg): ($t_{(8)}$=.169, n.s.)

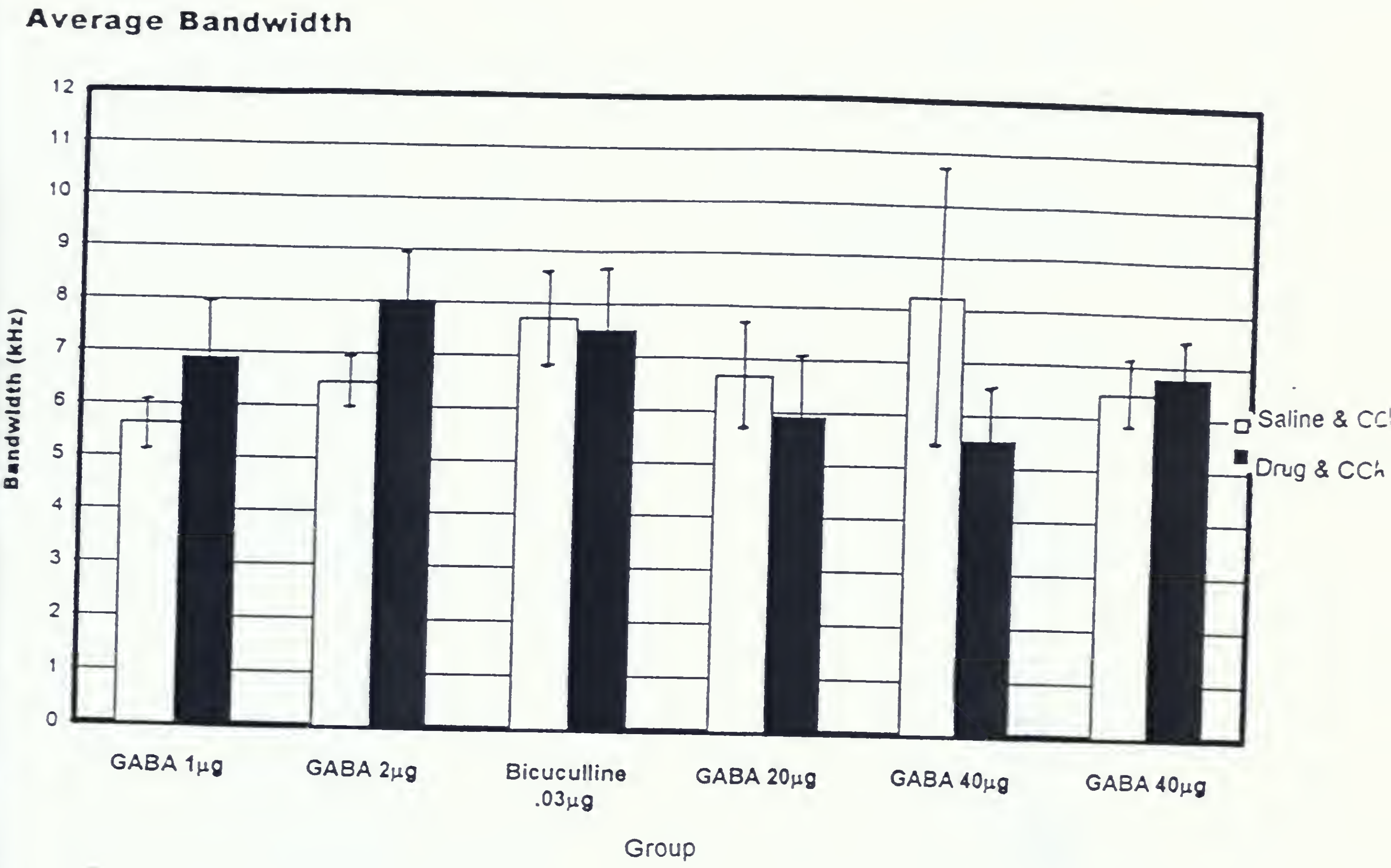

Figure 8. The effect of drug pre-treatment (GABA 1-40µg ic and bicuculline 0.03µg ic) on average bandwidth of CCh-induced vocalization. Each bar represents a mean bandwidth (kHz). Each pre-treatment was administered 2-5 minutes prior to injection of CCh (1.5µg in 0.2µl saline). There was no significant difference in average bandwidth between the control vehicle (blank bars) and the drug injections (filled bars). Vertical lines represent SEMs. Statistical results are as follows:

Group1 (GABA 1 µg): ($t_{(7)}=-1.024$, n.s.), group 2 (GABA 2 µg): ($t_{(5)}=-1.781$, n.s.), group 3 (bicuculline): ($t_{(7)}=.311$, n.s.), group 4 (GABA 20 µg): ($t_{(5)}=.456$, n.s.), group 5 (GABA 40 µg): ($t_{(1)}=1.577$, n.s.), group 6 (GABA 40 µg): ($t_{(8)}=-.472$, n.s.)

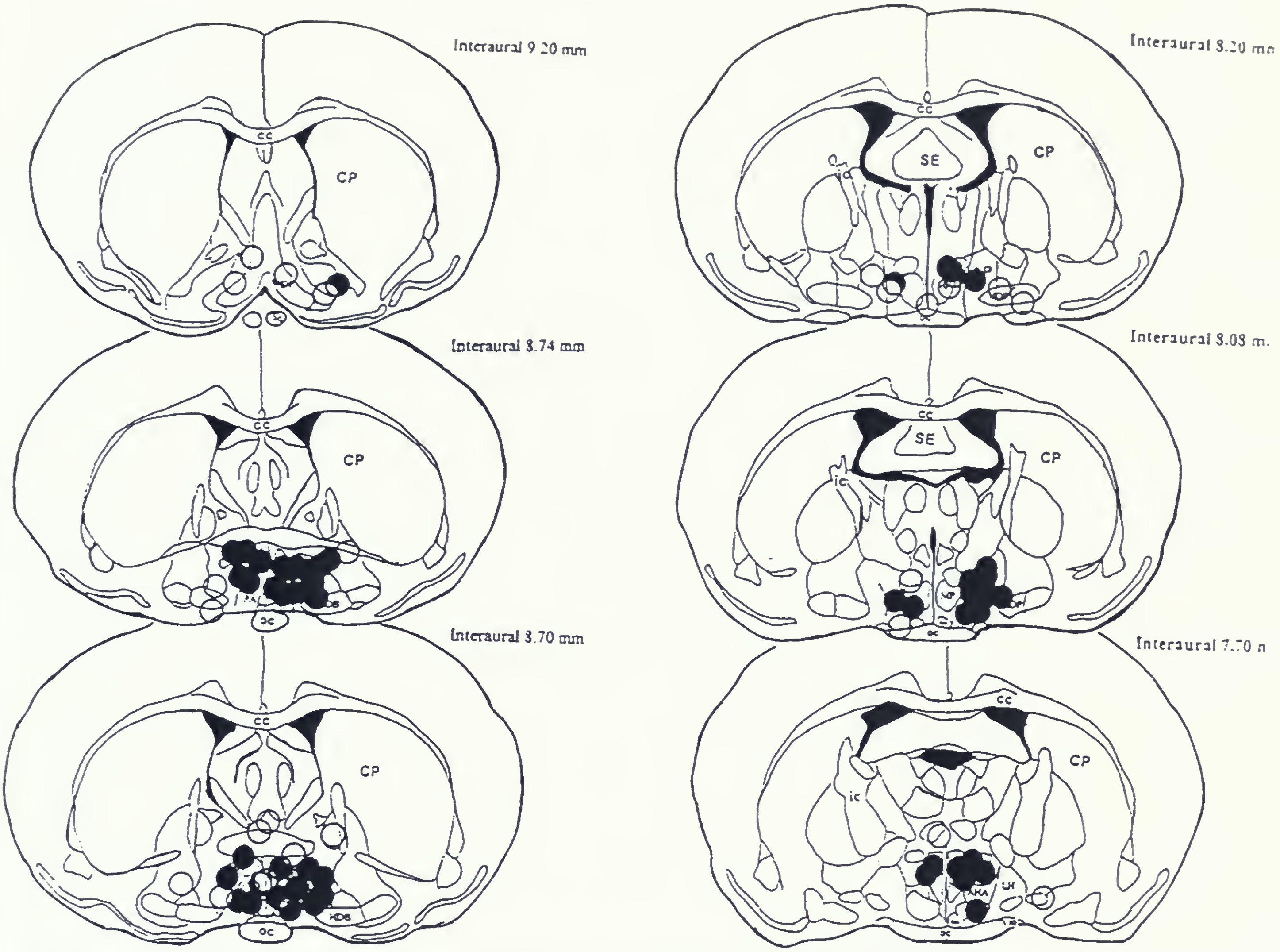

Figure 9. Localization of injection sites for pre-treatment and CCh in the AH/POA of the rat brain (circles) shown on frontal sections (A=9.20 mm, 8.74 mm, 8.7 mm, 8.2 mm, 8.08 mm, and 7.70 mm). Injection sites from which ultrasonic vocalization was induced consistently are marked with filled circles. Injection sites from which vocalization was induced from at least one injection but not all injections are marked with half-filled circles. Injection sites from which no ultrasonic vocalization was induced are marked with unfilled circles (histological verification indicates that sites not eliciting vocalization were out of range of the target area). Abbreviations: ac, anterior commissure; AHA, anterior hypothalamic area; cc, corpus callosum; CP, caudate-putamen; DB, horizontal limb of the diagonal band; fx; fornix; ic, capsula interna; LA, lateral preoptic area; LH, lateral hypothalamic area; MP, medial preoptic area; Oc, optic chiasm; PA, paraventricular hypothalamic nucleus; Sch, suprachiasmatic nucleus; SE, septum; TH,