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European Journal of Pharmacology 463 (2003) 133–143
www.elsevier.com/locate/ejphar

Stress-induced vocalisation in adult animals. A valid model of anxiety?

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Accepted 10 December 2002

Abstract

The post-stimuli anticipatory vocalisations that follow stressful and painful conditions are suggested as a quantitative measure of the emotional state of fear and anxiety in animal models. Adult rats emit characteristic 22-kHz ultrasound vocalisations consisting of 20–30 kHz calls with a mean duration of 300–600 ms as response to aversive stimuli (e.g. inescapable electric footshock, acoustic or air-puff stimuli, agonistic encounter or withdrawal from treatment with drugs of abuse). The vocalisations are accompanied by defensive submissive behaviour and signal a refractory, socially withdrawn or helpless state. Furthermore, brain structures that are involved in the mediation of anxiety-like behaviour, e.g. the dorsal periaqueductal grey and cortical areas, are also important for modulation of ultrasonic vocalisation. Benzodiazepines, e.g. diazepam, inhibit shock-induced ultrasonic vocalisation although the active doses are generally close to those that produce sedation and muscle relaxation. Selective serotonin reuptake inhibitors and other antidepressants that preferentially enhance serotonergic neurotransmission inhibit footshock-induced ultrasonic vocalisation. The 5-HT_2 receptor antagonistic properties of fluoxetine may explain why only partial inhibition is achieved. The biphasic dose–response curve of the racemic drug, citalopram, may perhaps be ascribed to an attenuating effect of *R*-citalopram. Tricyclic antidepressants, e.g. imipramine, and antidepressants that preferentially enhance catecholaminergic neurotransmission, e.g. reboxetine and venlafaxine, are inactive. Classical antipsychotics like haloperidol have no or a weak inhibitory effect. Serotonin plays a major role in the mediation of ultrasonic vocalisation, and in particular 5-HT_{1A} and 5-HT_2 receptors are found to have a prominent role. Different serotonergic pathways are likely to be involved in the mediation of the anxiolytic-like response, e.g. the pathway ascending from the dorsal raphe nucleus through the medial forebrain bundle to the amygdala and frontal cortex mediating conditioned/learned anxiety and another pathway ascending from the dorsal raphe nucleus to the periaqueductal grey mediating unconditioned/fight flight anxiety. Dopamine D_2 receptor agonists are potent inhibitors of footshock-induced ultrasonic vocalisation. The role of dopamine D_1 receptors and adrenoceptors remains to be further elucidated. Several other neurotransmitters are involved in the mediation of ultrasonic vocalisation, e.g. acetylcholine, histamine and glutamate. There is also a need for further studies of how changes in stress-axis function may modulate ultrasonic vocalisation and for studies of the effects of chronic drug treatment on ultrasonic vocalisation.

Keywords: Vocalisation; Stress; Aversion; Anxiety; Ultrasound; (Rat)

1. Introduction

Stressful and painful stimuli produce a series of behavioural and physiological responses in animals, often including intense vocalisation. Consequently, assessment of vocalisation is a common measure in many pharmacological and ethological studies of stress. The vast majority of these studies have been conducted in rodents, in particular in rats. The vocalisation pattern of rats is complex, with mainly audible vocalisation during application of the stimulus and post-stimulus vocalisations consisting of audible and ultrasonic frequencies, the so-called vocalisation afterdischarge response (Paalzow and Paalzow, 1975). The latter vocalisation is assumed to reflect the accompanying emotional state of fear and anxiety after the aversive stimulus. Furthermore, animals that have experienced an aversive stimulus previously will often exert conditioned, anticipatory vocalisation upon re-exposure to the same environment (e.g. review by Miczek et al., 1995).

Since vocalisation is an objective and relatively easy quantifiable measure, a considerable number of studies have been dedicated to validate stress-induced emotional vocalisation, in particular ultrasonic vocalisation in the rat, as animal models of anxiety. The value of conditioned, anticipatory and/or post-stimulation ultrasonic vocalisation for prediction of the anxiolytic potential of pharmacological agents has been given much attention by academia as well

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doi:10.1016/S0014-2999(03)01277-9

as the pharmaceutical industry over the last two to three decades. Drug-induced reduction of stress-induced ultrasonic vocalisation was suggested as a measure of anxiolytic activity by different researchers in the mid-1980s and in the beginning of the 1990s (Tonue et al., 1986; Cuomo et al., 1988; Hård and Engel, 1988; Kaltwasser, 1990; De Vry et al., 1993; Sánchez, 1993). Vocalisation is a prominent feature in rat pups as a response to separation and cold stress, and there are a vast number of studies addressing this condition as a model of anxiety. However, the present review will selectively address the value of stress-induced ultrasonic vocalisation in adult rats as model of anxiety. Rat pup models are discussed elsewhere.

The review will include a survey of the brain structures and neurotransmitter systems involved in the mediation of ultrasonic vocalisation and the accompanying behavioural characteristics and neuroendocrine changes, but will mainly focus on the utility of stress-induced ultrasonic vocalisation for prediction of the anxiolytic potential of new chemical entities.

2. Ultrasonic vocalisation and behavioural correlates in rats

In addition to vocalisation in the audible frequency range, rats hear and emit ultrasound vocalisations above the audible frequency range, and the communicative function and behavioural correlates of ultrasonic vocalisation have been studied extensively for more than 30 years (e.g. reviews by Sales and Pye, 1974; Sales et al., 1986). Different types of emotional states and behaviour changes have been related to two characteristic ultrasonic vocalisation frequency intervals in rats, the so-called 22- and 50-kHz vocalisations (Miczek et al., 1995). The former consists of 20–30-kHz calls with a mean duration of 300–600 ms and is characteristic for aversion-induced ultrasonic vocalisation. Whereas the stimulus-evoked audible vocalisation is sensitive to opiates, the anticipatory and post-stimulus vocalisations are sensitive to opiates and benzodiazepine anxiolytics (Van der Poel et al., 1989). The 22-kHz calls are emitted following noxious or aversive stimuli and are accompanied by defensive behaviour, by defeated submissive rats during agonistic encounters, in chronic pain conditions and in the refractory post-ejaculatory period (e.g. Sales, 1972; Sales and Pye, 1974; Anisko et al., 1978; Nyby and Whitney, 1978; Corrigan and Flanelly, 1979; Calvino et al., 1996; Brudzynske, 2001). These observations suggest that the 22-kHz calls signal a refractory, socially withdrawn or helpless state and that the emotional state of the animal could be reflected by the degree of vocalisation. In addition to the communicative value, emission of ultrasound vocalisations may also form part of a thermoregulatory behaviour. The 22-kHz ultrasonic vocalisation produces selective cooling of the hypothalamus and thereby reversal of stress-induced hyperthermia (Blumberg and Moltz, 1987).

The 50-kHz vocalisation consists of 50–70-kHz calls with a mean duration of less than 60 ms. Rats emit these calls in relation to rewarding stimuli and appetitive social encounters, such as mating and during an encounter with an intruder, and young rats use the calls in anticipation of play (Sales and Pye, 1974; Knutson et al., 1998). Rat pups emit 50-kHz calls during separation from their litter (Sales and Pye, 1974), but it has been suggested that these calls correspond to the 22-kHz emitted by the adult rats (Blumberg and Alberts, 1991).

3. Anatomical structures involved in stress-induced ultrasonic vocalisation in rats

As in most other mammals, the audible sounds are produced by vibrating structures in the larynx, while ultrasonic vocalisation is produced by a whistle-like mechanism in the larynx by expiring against a closed glottis (Roberts, 1975; Nyby and Whitney, 1978).

The major functions of the midbrain periaqueductal grey include pain and analgesia, fear and anxiety, vocalisation, copulatory behaviour (lordosis and mounting) and cardiovascular control (review by Behbehani, 1995). Electrical or chemical (e.g. kainic acid) stimulation of the dorsolateral periaqueductal grey produces marked flight responses, increased defensive reactions (e.g. upright postures, locomotion and jumps and freezing behaviour), 22-kHz calls and cardiovascular and respiratory stimulation (e.g. Yajima et al., 1980; Depaulis et al., 1992; review by Behbehani, 1995). These findings suggest that the dorsal periaqueductal grey is important for modulation of ultrasonic vocalisation as well as anxiety-like behaviour. In contrast, stimulation of the ventrolateral region of periaqueductal grey results in hyporeactivity and decreased cardiovascular activity (Depaulis et al., 1994).

A number of nuclei located in the lateral and dorsomedial part of the medulla oblongata (e.g. the lateral reticular nucleus and the facial nucleus) are also involved in the mediation of the 22-kHz ultrasonic vocalisation (Yajima et al., 1981).

Furthermore, cortical areas are likely to be involved in the mediation of stress-induced ultrasonic vocalisation. Air puff-induced 22-kHz calls were accompanied by significantly increased expression of Fos-like proteins in the medial prefrontal cortex, claustrum, cingulated cortex and dorsal central grey (Knapp et al., 1998). Lesion studies also suggest that cortical areas are involved, although their role to some extent remains unclear. Lesion of the ventral medial frontral cortex in adult rats reduced ultrasonic vocalisation and freezing behaviour significantly in a conditioned emotional response paradigm (Frysztak and Neafsey, 1991). Neonatal cerebral cortical lesion did not influence the production of footshock-induced ultrasonic vocalisation in the adult rat; however, diazepam failed to inhibit ultrasonic vocalisation in the lesioned animals, suggesting that this

mechanism is dependent on an intact cortical function (Naito et al., 1995).

4. Ultrasonic vocalisation models of anxiety in adult rats

Models in adult rats have been developed using various types of stressful stimuli, including inescapable electric footshock, acoustic or air-puff stimuli, agonistic encounter or withdrawal from treatment with drugs of abuse.

A considerable number of investigations have applied inescapable footshocks as aversive stimulus, and the majority of studies that address the pharmacological mechanisms have applied this principle (e.g. De Vry et al., 1993; Sánchez, 1993; Molewijk et al., 1995; Bartoszyk, 1998; Millan et al., 2000; Groenink et al., 1996). In these models, there is initial priming to the test condition followed by test sessions after drug treatment, although the number and intensity of shocks and inter-shock intervals and selection criteria vary.

Tactile stimulus is another reliable method for inducing 22-kHz vocalisation. It may consist of a short series of air puffs directed at the dorsal or dorsolateral part of the rat's head (e.g. Knapp and Pohorecky, 1995). Exposure to acoustic stimuli (e.g. a short series of 105–115-dB stimuli) produces startle responses and ultrasonic vocalisation in male rats (e.g. Kaltwasser, 1990, 1991), a response that is sensitive to anxiolytic compounds.

Intruder rats exposed to the resident's attacks and threats emit 22- as well as 50-kHz calls (Vivian and Miczek, 1993a,b). The former is reduced by gepirone whereas diazepam decreases the latter, possibly due to its muscle relaxant properties. The presence of a predator, e.g. a cat, in a rat colony will also result in 22-kHz alarm calls, calls which are reduced by treatment with morphine (Blanchard et al., 1991; Shepherd et al., 1992).

Chemically induced conditioned taste aversion may also produce 22-kHz calls, i.e. rats subjected to LiCl or naloxone-induced conditioned taste aversion have increased 22-kHz and decreased 50-kHz calls (Burgdorf et al., 2001b).

Finally, the increased rate of 22-kHz vocalisation during withdrawal from prolonged treatment with drugs of abuse is suggested to model the psychic anxiety related to this condition. Withdrawal from prolonged exposure (30–60 days) to moderate doses of oral cocaine produced a significant increase in tactile (air puffs)-induced ultrasonic vocalisation, but no increase in startle reflexes (Barros and Miczek, 1996). Rats withdrawn from an i.v. cocaine self-administration paradigm showed increased startle and ultrasonic vocalisation responses (Mutschler and Miczek, 1998). Similarly, withdrawal from exposure to ethanol resulted in increased spontaneous and air puff-induced 22-kHz vocalisation and physical withdrawal symptoms (e.g. tremor, convulsions and stereotypy), which were treatable with diazepam (Knapp et al., 1993, 1998). However, there is no simple relationship between an elevated state of emotionality, measured as 22-kHz vocalisation in response to tactile stimuli (air puff), and ethanol intake (Knapp et al., 1997). Startle response and 22-kHz vocalisation are also markedly increased 24 h after discontinuation of a 5-day diazepam treatment (2.5–10 mg/kg, i.p., b.i.d.), which is reduced by treatment with gepirone or diazepam (Miczek and Vivian, 1993; Vivian et al., 1994).

5. Pharmacological validation of stress-induced ultrasonic vocalisation. Acute studies

Most pharmacological studies have been conducted using footshock as aversive stimulus, and the outcome for a representative selection of pharmacological agents are summarised in Tables 1–4. Information on drug effects after other aversive stimuli is included in the text, when possible.

5.1. Psychotropics

5.1.1. Benzodiazepine anxiolytics

The effects of benzodiazepines seem to vary somewhat and are overall not very pronounced (Table 1). Diazepam is found to inhibit shock-induced ultrasonic vocalisation in most laboratories, although the active doses are generally

Table 1
Effect of benzodiazepine anxiolytics, selective reuptake inhibitors and other antidepressants and antipsychotics on footshock-induced vocalisation

Receptor/site	Compound	Footshock-induced 22-kHz vocalisation (mg/kg)		
		ED_{50}[a]	ED_{50}[b]	MED[c]
Benzodiazepines	alprazolam			3.0
	chlordiazepoxide	18[d]	>20	>10
	diazepam	2.9	3.5[b]	30
	zolpidem		5.2	>2.0
Selective serotonin reuptake inhibitors	citalopram	6.5	[e]	
	escitalopram		0.62[b]	
	fluoxetine	>30	>20[b]	
	fluvoxamine	11.7	7.3[b]	3.0
	paroxetine	6.9	0.32[b]	
	sertraline		6.7[b]	
Other antidepressants	imipramine	approximately 30	>9	10
	clomipramine	approximately 30	0.35	10
	mirtazepine		2.3	
	reboxetine		>7.2	
	venlafaxine		>18	
Antipsychotics	haloperidol	>10[d]	1.5	3.0
	clozapine	1.0	0.29	

Compounds were administered s.c. or i.p. unless stated otherwise.

[a] De Vry et al. (1993) and Schreiber et al. (1998).

[b] Sánchez and Meier (1997) and Sánchez et al. (1995 and 2003). Doses expressed as mg/kg base.

[c] Molewijk et al. (1995). Minimal effective doses, using reduction of number of calls as effect measure.

[d] p.o. administration.

[e] Biphasic response with maximum 64% inhibition at 0.63 mg/kg. ED_{50} calculated only on descending part of the dose–response curve reveals 0.41 mg/kg.

Table 2
Effect of 5-HT receptor ligands on footshock-induced vocalisation

Receptor/site	Compound	Footshock-induced 22-kHz vocalisation (mg/kg)		
		ED_{50}[a]	ED_{50}[b]	MED[c]
5-HT_{1A}	8-OH-DPAT	0.02	0.017[b]	0.03
Agonists	buspirone	2.0	0.16[b]	1.0
	ipsapirone	0.2	0.18[b]	3.0
	pindolol		1.2[b]	
Antagonist	WAY 100635		>20[b]	
5-HT_{1B}	TFMPP	1.8	0.39[b]	
Agonists	MCPP	1.4	0.12[b]	
Antagonist	GR 127935	>30	>18	
5-HT_2	DOI	1.4	0.39[b]	
Agonist				
Antagonists	ritanserin	>10	>2.5[d]	
	MDL 100151		>10[b]	
5-HT_3	ondansetron	>0.01	>0.13[b]	>0.1
Antagonist	ICS 205930	>1.0	>10[b]	

Compounds were administered s.c. or i.p. unless stated otherwise.
[a] De Vry et al. (1993) and Schreiber et al. (1998).
[b] Sánchez (1993), Sánchez and Meier (1997) and Sánchez et al. (1996). Doses are expressed as mg/kg base.
[c] Molewijk et al. (1995). Minimal effective dose, using reduction of number of calls as effect measure.
[d] Significantly increased ultrasonic vocalisation.

close to those that produce sedation and muscle relaxation (Tonue et al., 1986, 1987; Cuomo et al., 1988; Vivian et al., 1994; Molewijk et al., 1995; Bartoszyk, 1998; Table 1). The effects of a number of other benzodiazepines, e.g. chlordiazepoxide, are less consistent (Table 1; Van der Poel et al., 1989; Molewijk et al., 1995; De Vry et al., 1993). Aversion-induced ultrasonic vocalisation has been suggested to mimic panic-like anxiety (Molewijk et al., 1995), which in general is not well treated with benzodiazepines, except for alprazolam. The latter compound was also found to be more active than other benzodiazepines against ultrasonic vocalisation in one study (Molewijk et al., 1995).

Table 3
Effect of noradrenergic and dopaminergic ligands on footshock-induced ultrasonic vocalisation

Receptor/site	Compound	Footshock-induced 22-kHz vocalisation (mg/kg)			
		ED_{50}[a]	ED_{50}[b]	ED_{50}[c]	ED_{50}[d]
Noradrenaline α_1 agonist	ST 587		>2.5[b]		
Antagonist	prazosin	>0.30	0.16[b]		
Noradrenaline α_2 agonist	clonidine	Approx. 0.3[e]	0.085[b]	0.02	
	dexmedetomidine		0.011	0.005	
Antagonist	idazoxan	0.9	0.51		
	yohimbine	0.9	0.13		
Noradrenaline β agonist	clenbuterol		>8.9[b]		
Antagonist	metoprolol		>15[b]		
Dopamine D_1 agonist	SKF 38393		>8.7		>3.0
Antagonist	SCH 23390		[f]		>3.0
Dopamine D_2 agonist	apomorphine		0.029		0.07
	quinpirole		0.015		0.04
	roxindole		0.018		0.04
	talipexole		0.065		0.04

Compounds were administered s.c. or i.p. unless otherwise stated.
[a] De Vry et al. (1993) and Schreiber et al. (1998).
[b] Sánchez (1993). Doses are expressed as mg/kg base.
[c] Millan et al. (2000).
[d] Bartoszyk (1998).
[e] p.o. administration.
[f] Biphasic response, significantly increased ultrasonic vocalisation at 0.08 and 0.02 mg/kg, no effect at 0.31 mg/kg.

Table 4
Inhibition of footshock-induced ultrasonic vocalisation, effects of other neurotransmitter systems

Receptor/site	Compound	Footshock-induced 22-kHz vocalisation ED_{50} (mg/kg)[a]
Acetylcholine	scopolamine	0.088
Muscarinic	atropine	12
GABA	tiagabine	>9.0
	gaboxadol	>7.8
	FG 7142	8.1
Histamine	mepyramine	6.6
NMDA	MK 801	0.095
	CPP	>4.8
Opiate	morphine	3.9
	naloxone	>18

Compounds were administered s.c. or i.p. unless otherwise stated.
[a] Doses are expressed as mg/kg base.

5.1.2. Antidepressants

The selective serotonin reuptake inhibitors have gained extensive clinical use during the last two decades and are drugs of choice for the treatment of depressive and anxiety disorders. Selective serotonin reuptake inhibitors antagonise footshock-induced ultrasonic vocalisation (Table 1), although the inhibitory effect of fluoxetine is only partial and citalopram produces a biphasic dose–response curve. These differences may suggest quantitatively different pharmacological profiles within this drug class. Fluoxetine has notable 5-HT_2 receptor antagonistic properties, and it may be that these properties attenuate the effect of fluoxetine against ultrasonic vocalisation (see discussion of this mechanism in Section 5.2).

Citalopram is a racemic mixture of *S*-(+)- and *R*-(−)-enantiomers (escitalopram and *R*-citalopram, respectively) in a 1:1 ratio, and escitalopram has recently been developed for clinical use to treat major depression and anxiety disorders (Montgomery et al., 2001; Burke et al., 2002; Wade et al., 2002). The 5-HT reuptake inhibitory activity of citalopram has been reported to reside in the *S*-enantiomer (Hyttel et al., 1992; Sánchez et al., in press). However, unlike citalopram, escitalopram was able to inhibit footshock-induced ultrasonic vocalisation completely. *R*-Citalopram also partially inhibited footshock-induced ultrasonic vocalisation, but was several times less potent than escitalopram and citalopram (Sánchez et al., 2003). The partial inhibition produced by citalopram and

the biphasic nature of its dose–response could be because *R*-citalopram exerts a dampening effect on the action of escitalopram as the basal level of 5-HT increases. This hypothesis is supported by recently presented data from a microdialysis study where *R*-citalopram was found to attenuate escitalopram-induced increase in the 5-HT output in the prefrontal cortex of freely moving rats (Mørk et al., 2002). These findings are currently being investigated in further detail.

The potency of paroxetine is remarkably different, more than 20-fold, in studies conducted by Schreiber et al. (1998) and Sánchez and Meier (1997). This is not readily explained, as there is not a general difference in the potency of other drugs between the two laboratories (Tables 1–4). One possible explanation could relate to the different response to cholinergic antagonists, i.e. no effect and a potent inhibition of ultrasonic vocalisation ($ED_{50} = 0.088$ mg/kg in Table 4), respectively. Paroxetine has in vivo anticholinergic activity that is shown as antagonism of oxotremorine-induced hypothermia in the milligram per kilogram dose range (Sánchez, 2002). It cannot be excluded that this effect contributes to the inhibition by paroxetine of ultrasonic vocalisation in our model.

Unlike selective serotonin reuptake inhibitors and other antidepressants that preferentially enhance serotonergic neurotransmission (e.g. clomipramine in Table 1), tricyclic antidepressants, e.g. imipramine, and antidepressants preferentially enhancing catecholaminergic neurotransmission, e.g. reboxetine and venlafaxine, are generally not very active against footshock-induced ultrasonic vocalisation (Tables 1 and 2). Mirtazepine has a rather potent effect against ultrasonic vocalisation, and both serotonergic and noradrenergic receptors may be involved in mediating this effect.

5.1.3. Antipsychotics

Classical antipsychotics like haloperidol have no or a weak inhibitory effect on footshock-induced ultrasonic vocalisation, only being active at doses that induce catalepsy. This is in line with the limited anxiolytic activity of these drugs in the clinic. In contrast, clozapine, the prototype of so-called atypical antipsychotics, which selectively inhibits limbic rather than striatal dopaminergic activity, had a marked effect (Table 1). However, this is not a common feature of limbic-selective antipsychotics, e.g. sertindole is devoid of activity in this paradigm (Sánchez et al., 1995).

5.2. 5-HT receptors

Serotonin plays a major role in the mediation of ultrasonic vocalisation, e.g. marked effects of selective serotonin reuptake inhibitors and the 5-HT releasing agent, fenfluramine (Sánchez, 1993; Sánchez and Meier, 1997). At least 16 different 5-HT receptor subtypes have been identified (reviewed by Hoyer et al., 2002), but their role in the mediation of ultrasonic vocalisation remains unclear for most of these receptor subtypes, partly because of a lack of selective pharmacological compounds.

5-HT_{1A} receptor agonists (e.g. 8-hydroxy-dipropyltetraline [8-OH-DPAT], buspirone, gepirone and ipsapirone) are consistently found to inhibit ultrasonic vocalisation induced by various types of aversive stimuli, suggesting a prominent role of these receptors in the mediation of 22-kHz calls (Rowan et al., 1990; Kaltwasser, 1991; De Vry et al. 1993; Sánchez, 1993; Molewijk et al., 1995; Table 2). Pre-synaptic 5-HT_{1A} receptors situated on serotonergic cell bodies in the dorsal raphe nucleus rather than post-synaptic receptors in the cortical and hippocampal terminal areas are thought to be involved. Thus, systemic administration of the 5-HT_{1A} receptor antagonist, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide (WAY 100635), reversed 8-OH-DPAT (systemic or local administration)-induced inhibition of footshock-induced ultrasonic vocalisation, whereas local application of 8-OH-DPAT into the hippocampus only produced inhibition at doses that were higher than those that were active after i.v. administration (Remy et al., 1996; Jolas et al., 1995). This is consistent with the reduced firing rate of serotonergic neurons in the dorsal raphe nucleus and with the reduced 5-HT output in terminal areas, e.g. hippocampus, after systemic administration of ipsapirone (Sommermeyer et al., 1993). However, 5-HT_{1A} receptor agonists, e.g. 8-OH-DPAT, also inhibit periaqueductal grey neuronal activity in the rat in both in vivo and in vitro studies, suggesting that this brain structure also plays an important role in the 5-HT_{1A} receptor agonist-induced inhibition of ultrasonic vocalisation (Behbehani et al., 1993).

Reduced 5-HT_{1A} receptor activity, produced by treatment with the receptor inactivating compound, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), or the 5-HT-depleting agent, *p*-chloro-phenylalanine methyl ester, and stimulation of serotonin receptors, produced by treatment with a 5-HT_{1A} receptor agonist, or an overall increase in serotonin neurotransmission produced by treatment with a selective serotonin reuptake inhibitor or a 5-HT releasing agent, fenfluramine, all result in reduced ultrasonic vocalisation. This may be in line with the observation that different serotonergic pathways are involved in the mediation of the anxiolytic-like response in this paradigm (Sánchez and Mørk, 1999). These observations agree with the dual 5-HT fear hypothesis by which two different pathways mediate conditioned/learned and unconditioned/fight flight anxiety, respectively (Graeff et al., 1996). Both types of anxiety are likely to be components of a response to a stimulus in a previously experienced context. The former type of anxiety is suggested to be mediated by the ascending 5-HT pathway from the dorsal raphe nucleus through the medial forebrain bundle to the amygdala and frontal cortex, whereas the latter pathway is suggested to be mediated from the dorsal raphe nucleus to the periventricular and periaque-

ductal grey matter. According to this hypothesis, the consequences of an increased availability of 5-HT are opposite for the two pathways, i.e. increased conditioned anxiety mediated by stimulation of post-synaptic 5-HT receptors in the amygdala and decreased unconditioned (fight/flight) anxiety mediated by stimulation of 5-HT receptors in the periaqueductal grey. However, the relative role of these pathways and the involvement of different subtypes of 5-HT receptors in mediating ultrasonic vocalisation need to be studied in further detail.

Even 5-HT_{1A} receptor agonists with low intrinsic activity are potent inhibitors of ultrasonic vocalisation. Thus, pindolol, a β-adrenoceptor antagonist and 5-$HT_{1A/1B}$ receptor low efficacy agonist, which is given in combination with selective serotonin reuptake inhibitors to improve clinical efficacy and/or onset to action, inhibits ultrasonic vocalisation (Sánchez et al., 1996). The improved antidepressant effect of selective serotonin reuptake inhibitors given in combination with pindolol is normally ascribed to an antagonistic effect at pre-synaptic 5-HT_{1A} receptors situated on the serotonin neuron cell bodies. But it cannot be excluded that the 5-HT_{1A} receptor agonistic properties of pindolol contribute to its effects on anxiety and depression symptoms as well.

The predicted anxiolytic potential of 5-HT_{1A} receptor agonists in aversion-induced ultrasonic vocalisation is supported by the fact that buspirone is used in the clinic for the treatment of psychiatric conditions involving anxiety symptoms. However, the effect of buspirone is generally perceived as being rather weak and the outcome of clinical trials with other 5-HT_{1A} receptor agonists (e.g. ipsapirone and flesinoxan) has been rather disappointing. The selective serotonin reuptake inhibitors appear to be a better alternative for the treatment of anxiety symptoms; possibly underlining that activation of various 5-HT receptor subtypes is a more efficacious therapeutic approach.

The 5-$HT_{1B/2C}$ receptor agonist 1-(3-trifluoromethylphenyl)piperazine (TFMPP) abolishes footshock-induced ultrasonic vocalisation, probably through 5-HT_{1B} receptor stimulation because the effect was reversed by the 5-$HT_{1A/1B}$ receptor antagonist, (−)-penbutolol, which is devoid of affinity for 5-HT_{2C} receptors, and the selective 5-HT_{1A} receptor antagonist, WAY 100635, was inactive (Table 2; Sánchez et al., 1996).

5-HT_{2A} receptor agonists, e.g. 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), inhibit ultrasonic vocalisation, suggesting that these receptors are involved in the mediation of the vocal response, too (Table 2). This is supported by the finding that the 5-HT_2 receptor antagonist, ritanserin reversed 5-hydroxytryptophan (5-HTP)-induced inhibition of ultrasonic vocalisation (Sánchez and Mørk, 1999). Furthermore, selective serotonin reuptake inhibitor-induced antagonism of ultrasonic vocalisation was reversed by the selective 5-HT_{2A} receptor antagonist, (+)α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorphenyl)ethyl]-4-piperidinmethanol (MDL 100907), but not by a 5-HT_{1A} receptor antagonist, WAY 100635, or a 5-HT_{1B} receptor antagonist, *N*-[methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide (GR 127935) (Schreiber et al., 1998). Actually, ritanserin produced a significant increase in ultrasonic vocalisation in one study, suggesting an anxiogenic-like effect (Sánchez, 1993). A receptor inactivation study with EEDQ showed that protection of 5-HT_2 but not 5-HT_{1A} receptors by pre-treatment with MDL 100907 and WAY 100635, respectively, was sufficient for preserving the ultrasonic vocalisation response (Sánchez and Mørk, 1999). This also supports a role for 5-HT_2 receptors in mediation of ultrasonic vocalisation.

5-HT_3 receptor antagonists are inactive against ultrasonic vocalisation (Table 2). A large number of 5-HT_3 receptor antagonists were presented as putative anxiolytics during the late 1980s (e.g. reviewed by Costall and Naylor, 1991). They were reported to be particularly potent in the black and white two-compartment test and in the social interaction test. Later reports have been less consistent and clinical studies failed to show anxiolytic activity (Schneier et al., 1996).

5.3. Catecholamines

5.3.1. Noradrenaline

Stress activates the sympathic nervous system, resulting in increased cardiovascular and catecholaminergic activity. Our understanding of the role of adrenoceptors in the mediation of ultrasonic vocalisation is, however, rather incomplete. Increased noradrenergic neurotransmission following treatment with a selective noradrenaline reuptake inhibitor, desipramine, tends to increase the number of calls, although the effect did not reach statistical significance (Molewijk et al., 1995). A similar trend has been observed with another selective noradrenaline reuptake inhibitor, talsupram ($p = 0.06$; unpublished observation). In line with this, attenuation of noradrenergic neurotransmission by treatment with the pre-synaptic acting α_2-adrenoceptor agonist, clonidine, or the post-synaptic acting α_1-adrenoceptor antagonist, prazosin, reduced footshock-induced ultrasonic vocalisation (Table 3). However, clonidine was found to intensify ultrasonic vocalisation and defensive behaviour and to attenuate the tachycardic response in the resident intruder paradigm (Tornatzky and Miczek, 1994). A role of α_1-adrenoceptors in the mediation of ultrasonic vocalisation is supported by the finding that pre-treatment with ketanserin, which has high affinity for α_1-adrenoceptors, protected against EEDQ-induced receptor inactivation (unpublished observations). β-Adrenoceptor antagonists are sometimes prescribed for the treatment of stressful conditions although their effect may be questioned. They are consistently found to be inactive against stress-induced ultrasonic vocalisation (e.g. Table 3; Tornatzky and Miczek, 1994; Sánchez et al., 1996).

Rather unexpectedly the α_2-adrenoceptor antagonists, idazoxan and yohimbine, were also found to inhibit footshock-induced ultrasonic vocalisation (Table 3; De Vry et al., 1993; Molewijk et al., 1995). An anxiogenic-like response might have been expected because yohimbine is reported to have anxiogenic properties in humans. However, effects at other receptor types or some degree of intrinsic activity at α_2-adrenoceptors could also explain these findings. Alternatively, effects at 5-HT receptors may explain the effect because both yohimbine and idazoxan show affinity for 5-HT receptors.

5.3.2. Dopamine

The mixed dopamine D_1 and D_2 receptor agonist, apomorphine, increased the threshold for audible afterdischarge vocalisation induced by electric stimulation of the tail (Paalzow and Paalzow, 1975). More recently, it has become evident that dopamine D_2 and dopamine $D_{2/3}$ receptor agonists together with 5-HT_{1A} receptor agonists are among the most potent inhibitors of footshock-induced ultrasonic vocalisation, with ED_{50} values of a few micrograms per kilogram, suggesting that this mechanism may be of relevance for the treatment of anxiety conditions (Bartoszyk, 1998; Table 3). The high potencies suggest that pre-synaptic rather than post-synaptic dopamine D_2 receptors are involved in the inhibition of ultrasonic vocalisation. It could be argued that the motor sedation, which accompanies pre-synaptic stimulation of dopamine D_2 receptors, is responsible for the inhibition of ultrasonic vocalisation. However, this may not be the case as other sedating compounds, e.g. the β-adrenoceptor agonist, clenbuterol, and the γ-aminobutyric acid ($GABA_A$) receptor agonist, gaboxadol, are devoid of effect even at high doses (Tables 3 and 4). Stimulation of post-synaptic dopamine receptors with higher doses of dopamine D_2 receptor agonists may be a major drawback for the potential of these compounds as anxiolytics because of the risk of motor side effects and abuse potential. Dopamine D_1 receptor agonists are devoid of effect on footshock-induced ultrasonic vocalisation (Table 3).

Dopamine $D_{2/4}$ receptor antagonists (e.g. haloperidol and emonapride) do not affect footshock-induced ultrasonic vocalisation significantly (Table 1 and unpublished observation, respectively). In one study, the dopamine D_1 receptor antagonist, SCH 23390, increased ultrasonic vocalisation significantly, suggesting an anxiogenic-like activity (Table 3). The dose–response curve was bell-shaped, with a maximum increase of about 50% at 0.02 mg/kg. The mechanism of this effect is not understood, and the anxiogenic activity of dopamine D_1 receptor antagonists is not a well-established effect.

Inhibitors of dopamine uptake (e.g. GBR 12909 and indatraline) are inactive against 22-kHz calls, whereas the 50-Hz calls are increased by amphetamine (Bartoszyk, 1998; Wintink and Brudzynski, 2001). Amphetamine injection into the nucleus accumbens, a key area for mediation of arousal and expectation of reward, elicited 50-kHz vocalisation, supporting the notion that these calls are related to appetitive states (Burgdorf et al., 2001a,b).

5.4. Other neurotransmitter systems

Opioid receptor agonists inhibit ultrasonic vocalisation induced by aversive stimuli in most (Table 4, Tonue et al., 1986, 1987; Vivian and Miczek, 1993b) but not all studies (De Vry et al., 1993).

The histamine H_1 receptor antagonist, mepyramine, also antagonises ultrasonic vocalisation, suggesting that this neurotransmitter system is involved although its exact role remains to be elucidated. It may be an indirect effect because histamine and histamine receptors are known to modulate serotonergic neurotransmission.

Some studies suggest that muscarinic cholinergic receptors are involved in the mediation of 22-kHz ultrasonic vocalisation, e.g. the antagonist, scopolamine, inhibited footshock-induced ultrasonic vocalisation (Table 4) and local administration of the agonist, carbachol, into the anterior hypothalamic-preoptic area induced 22-kHz vocalisation, an effect which was reversed by atropine (Brudzynski, 1994). However, another study failed to show any inhibitory effect of scopolamine (up to 1 mg/kg) against footshock-induced ultrasonic vocalisation (De Vry et al., 1993), making the role of muscarinic acetylcholine receptors less clear.

Glutamate injected into the intrahypothalamic preoptic area of the rat has been found to increase the number of 50-kHz calls significantly and the effect was reversed by systemic treatment with the NMDA receptor antagonist, MK 801, or haloperidol (Fu and Brudzynski, 1994; Wintink and Brudzynski, 2001). However, glutamate is also involved in mediation of the 22-kHz calls because the non-competitive NMDA receptor antagonist, MK 801, inhibits footshock-induced ultrasonic vocalisation (Table 4; De Vry et al., 1993). The competitive NMDA receptor antagonist, (*R*,*S*)-3-(2-carboxypiperazin-4-yl)propyl)phosphonic acic (CPP), is not active (Table 4). Another line of evidence come from the observation that glutamatergic stimulation of the ascending cholinergic projections from the ponto-mesencephalic cholinergic nuclei resulted in 22-kHz calls and that these calls were antagonised by a scopolamine injection into the terminal areas in the mediobasal hypothalamic-preoptic region (Brudzynski and Barnabi, 1996).

Increased GABAergic neurotransmission, elicited by treatment with the GABA uptake inhibitor, tiagabine, or the $GABA_A$ receptor agonist, gaboxadol, did not affect footshock-induced ultrasonic vocalisation (Table 4). Picrotoxin and pentetrazol, both antagonists acting at modulatory binding sites on the $GABA_A$ receptor complex, were found to increase ultrasonic vocalisation significantly, indicating an anxiogenic-like effect (De Vry et al., 1993). The inverse benzodiazepine agonist, FG 7142, failed to increase ultrasonic vocalisation (De Vry et al., 1993) or reduced ultra-

sonic vocalisation at high doses (ED_{50} = 8.1 mg/kg). This lack of anxiogenic-like effect may be due to the fact that FG 7142 is a partial inverse agonist, and it may be that inverse agonists with higher intrinsic activity would produce an anxiogenic-like effect. Thus the anxiolytic/anxiogenic-like effects mediated by the $GABA_A$ receptor complex seems to involve modulatory sites on the receptor rather than the primary binding site.

6. Chronic studies

The extensive pharmacological validation of stress-induced ultrasonic vocalisation is based on acute studies, whereas chronic treatment is required to achieve a clinical effect in anxiety disorders. However, only a very limited number of chronic studies of stress-induced vocalisation have been reported.

In one study, the ability of diazepam (1.3 or 2.5 mg/kg b.i.d. for 3 weeks) to reduce footshock-induced ultrasonic vocalisation remained unchanged, whereas the duration of vocalisation gradually decreased in the vehicle control group, which makes interpretation of the results difficult (Nielsen and Sánchez, 1995). Ultrasonic vocalisation was not different in diazepam-treated compared to vehicle-treated rats 24 and 48 h after discontinuation, suggesting a lack of withdrawal anxiety in this paradigm. In another study, the anxiolytic-like effect of ipsapirone was found to be unchanged after prolonged treatment (Xu et al., 1997). The effect of daily injection of diazepam or clomipramine has also been studied during the extinction phase of a conditioned 22-kHz vocalisation paradigm (Kikusui et al., 2001). Rats were conditioned by daily exposure to electric footshocks for at least 10 days and subsequently tested daily during the extinction phase. Clomipramine (20 mg/kg, i.p., 45 min) but not diazepam (1 mg/kg, i.p., 30 min) reduced the number of sessions needed for extinction significantly compared to the effect of vehicle. In line with this, stress-induced ultrasonic vocalisation is suggested to model panic-like anxiety conditions where clomipramine and other serotonin-enhancing antidepressants are more effective than benzodiazepines.

7. Stress-induced vocalisation and stress-axis function

Various attempts have been made to correlate emotional responses to stress and changes in stress-axis function; however, the outcomes of these studies are often contradictory.

Conditioned emotional stress produced by unavoidable footshock increased 22-kHz ultrasonic vocalisation and plasma adrenocorticotropic hormone (ACTH), corticosterone, and prolactin levels in rats. The former but not the latter effect was reversed by treatment with the $5\text{-}HT_{1A}$ receptor antagonist, WAY 100635, suggesting that this receptor is involved in the behavioural but not the neuroendocrine response to emotional stress (Groenink et al., 1996).

Corticotropin-releasing factor (CRF), which plays a key role in the regulation of the endocrine responses to stress and produces anxiogenic effect when administered into the brain, was found to interfere in a complex manner with fear-conditioned ultrasonic vocalisation in rats (Kikusui et al., 2000). Thus, studies with the CRF antagonists, α-hCRF and CP-154,526, suggested that central CRF systems are involved in the retrieval process, but not the acquisition or retention process of fear-related memory (Kikusui et al., 2000).

Rats that were handled throughout the pre-weaning period showed less emotional reactivity, measured as a reaction to handling (i.e. startle response, vocalisation and resistance to being picked up), than non-handled controls and rats that had received footshocks during the pre-weaning period, but there was no correlation between emotional reactivity and changes in plasma corticosterone concentration (Ader, 1968). Similarly, a comparative study of the selectively bred Tsukuba High Emotional (THE) and Tsukuba Low Emotional (TLE) strains revealed that the high emotionality was reflected by in an increased emission of 22-kHz calls, but was not related to a different degree of activation of the hypothalamic–pituitary axis (Naito et al., 2001).

8. Emotional vocalisation by other species

Other rodents, e.g. mice, hamsters and gerbils, also communicate by means of ultrasonic vocalisation in relation to different behavioural contexts, although the frequency range used varies between species (Sales and Pye, 1974). Very few anxiety paradigms have been developed in adult animals for these species. Mongolian gerbils emit ultrasonic vocalisation at about 33 kHz in response to conspecifics and their body odours (Thiessen and Upchurch, 1981). Haloperidol and clonidine increased and apomorphine reduced the ultrasonic vocalisation.

A few pharmacological studies have also been conducted in non-rodent adult animals. For instance, separation-induced vocalisation by adult squirrel monkeys is decreased by imipramine (Harris and Newman, 1987). The effect is suggested to be mediated by α_2-adrenoceptors, as the α_2-adrenoceptor agonist, clonidine, reduced and the antagonist, yohimbine, increased the vocalisation, and yohimbine but not prazosin reversed the effect of clonidine.

9. Overall conclusion

Behavioural, physiological, neuroanatomical and pharmacological studies suggest that stress-induced vocalisation is a valid measure of the emotional status in many mammals. Rats have been the preferred species for these

investigations, and the long 22-kHz calls have shown to be a valuable measure of a defeated, submissive withdrawn emotional condition. Inhibition of aversion-induced ultrasonic vocalisation may therefore be a useful model for assessment of the anxiolytic potential of new chemical entities. However, a number of issues still remain to be clarified. The pharmacological characterisation suggests that several neurotransmitter systems are involved in the mediation of aversion-induced 22-kHz calls, although the relative role of these pathways remains to be studied in further detail. There is also a need for further studies of how changes in stress-axis function may modulate ultrasonic vocalisation. Finally, the utility of stress-induced ultrasonic vocalisation for studies of the effects of chronic drug treatment should be addressed in greater detail.

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