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Research paper

Differential effects of iontophoretic in vivo application of the $GABA_A$-antagonists bicuculline and gabazine in sensory cortex

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Abstract

We have compared the effects of microiontophoretic application of the $GABA_A$-receptor antagonists bicuculline (BIC) and gabazine (SR95531) on responses to pure tones and to sinusoidally amplitude-modulated (AM) tones in cells recorded extracellularly from primary auditory cortex (AI) of Mongolian gerbils. Besides similar effects in increasing spontaneous and stimulus-evoked activity and their duration, both drugs elicited differential effects on spectral tuning and synchronized responses to AM tones. In contrast to gabazine, iontophoresis of the less potent $GABA_A$-antagonist BIC often resulted in substantial broadening of frequency tuning for pure tones and an elimination of synchronized responses to AM tones, particularly with high ejecting currents. BIC-induced effects which could not be replicated by application of gabazine were presumably due to the well-documented, non-GABAergic side-effects of BIC on calcium-dependent potassium channels. Our results thus provide strong evidence that $GABA_A$-mediated inhibition in AI does not sharpen frequency tuning for pure tones, but rather contributes to the processing of fast temporal modulations of sound envelopes. They also demonstrate that BIC can have effects on neuronal response selectivity which are not due to blockade of GABAergic inhibition. The results have profound implications for microiontophoretic studies of the role of intracortical inhibition in sensory cortex.

Keywords: Primary auditory cortex; Spectral tuning; Temporal processing; Intracortical inhibition; SR95531

1. Introduction

γ-Aminobutric acid (GABA) is the major inhibitory neurotransmitter in cerebral cortex (Curtis and Johnston, 1974; Krnjević, 1984; Winer, 1992) where it acts mainly on $GABA_A$- and $GABA_B$-receptors (Bormann, 1988, 2000; Chebib and Johnston, 1999). The structure, distribution and functional role of $GABA_A$-receptors has been the subject of intense investigation (e.g., Bowery, 1983; Hevers and Luddens, 1998; for review see Egebjerg et al., 2002). These receptors mediate fast inhibitory postsynaptic potentials (Metherate, 1998; Li et al., 1996) which are particularly well-suited to generate or sharpen receptive field properties of cortical neurons. Following the pioneering studies of Sillito (1975a,b), the functional role of $GABA_A$-receptors in sensory cortex has been investigated extensively by iontophoretically applying the competitive $GABA_A$-receptor antagonist bicuculline (BIC) whilst monitoring the responses of extracellularly recorded cells. However, it has been known for many years that, in addition to blocking $GABA_A$-receptors, BIC has several

Abbreviations: AI, primary auditory cortex; AM, sinusoidally amplitude-modulated tones; BF, best frequency; BIC, bicuculline methiodide; FRR, frequency response range; GABA, γ-aminobutyric acid; $GABA_A$, GABA receptor type A; PSTH, peri-stimulus time histogram; Q, quality-value characterizing tuning sharpness; SD, standard deviation; SR95531, gabazine; tMTFs, temporal modulation transfer functions; VS, vector strength

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non-GABAergic side-effects which may affect neuronal discharges in a dose-dependent manner. These include inhibition of GABA uptake, reduction of resting membrane conduction resulting in membrane depolarization, prolongation of calcium-dependent action potentials, paroxysmal depolarization shifts and apamin-like potentiation of burst firing (Olsen et al., 1976; Heyer et al., 1981; Johnson and Seutin, 1997). At least some of these secondary effects are thought to result from actions of BIC on calcium-dependent potassium channels (Johansson et al., 2001). In contrast, the pyridazinyl-GABA derivative gabazine has a higher affinity for the $GABA_A$-receptor than BIC (Michaud et al., 1986) and does not seem to induce the non-GABAergic effects associated with the application of BIC (Chambon et al., 1985; Heaulme et al., 1986; Hamann et al., 1988). Despite this, gabazine has not been widely used as a $GABA_A$-antagonist in studies designed to assess the functional role of intracortical inhibition.

Here, we have compared for the first time the effects of microiontophoretic application of BIC and gabazine on the selectivity of cortical cells to the same stimuli. We studied drug-induced effects on frequency tuning for pure tones and temporal processing of AM tones in AI. We focused on these properties, because (i) studies which have employed iontophoretic application of BIC have yielded conflicting results on the contribution of GABAergic inhibition to frequency tuning for pure tones (Schulze and Langner, 1999; Wang et al., 2000, 2002; Foeller et al., 2001); and (ii) there is substantial evidence that GABAergic inhibition plays an important role in the temporal processing of sounds, and (iii) GABAergic inhibition has been implicated in phase-locking of responses to AM tones at the level of AI (Grothe, 2000; Grothe and Klump, 2000; Schulze et al., 2002).

The results clarify the contribution of $GABA_A$-mediated inhibition to the spectral and temporal processing of sounds in AI and demonstrate that iontophoretic application of BIC can have deleterious effect on neuronal response selectivity which do not reflect the loss of GABAergic inhibition. They cast doubt on the conclusions drawn from previous studies in which iontophoretic application of BIC has been used to elucidate the functional role of GABAergic inhibition in sensory cortex. Parts of this study have been published in abstract form (Kurt et al., 2004, 2005).

2. Materials and methods

Experiments were performed on anesthetized ($n = 8$) or unanesthetized ($n = 14$) adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 80–120 g.

2.1. Animal preparation

Animals were prepared according to procedures described in detail elsewhere (Schulze and Langner, 1997). Surgery was performed under deep general (halothane, Sigma) and local anesthesia (Gingicain, Aventis). Body temperature was maintained at 37 °C using a remote-controlled heating blanket. Prior to surgery, ear canals and the tympanic membranes were inspected and found to be free of disease. The left auditory cortex was exposed by craniotomy, leaving the dura intact.

For acute experiments on anesthetized animals, a 2.5 cm-long rectangular aluminum bar was fixed to the frontal bones with dental acrylic and served as a head anchor for stereotaxic fixation during recordings. Insect needles were inserted into the skull to improve the stability of the head anchor and served as reference electrodes. The animals were then transferred to an anechoic, sound-attenuated chamber. Throughout the experiments, anesthesia was maintained by an intraperitoneal infusion of ketamine (50 mg/ml; Ratiopharm), xylazine (Rompun 2%, BayerVital) and isotonic sodium chloride solution (mixture 9:1:10) at a rate of 0.06 ml/h. Body temperature was maintained at 37 °C. At the end of a recording session (which typically lasted 20–24 h), animals were killed by an intrapulmonary injection of T61 (Intervet).

For experiments on unanaesthetized animals, a chronic preparation was used. The stability of the head anchor was further improved by inserting a small metal pin into the exposed left bulla. A plastic cylindrical chamber (diameter 6.5 mm) with a screw cap was then attached to the skull over the trepanation area. Insect pins, bulla anchor, head holder and cylinder were fixed with dental acrylic, resulting in a weight of the whole construction of about 5 g. Following surgery, the dura was covered with an antibiotic paste (Volon, Dermapharm, Grünwald) and the plastic cylinder was sealed with the screw cap. Animals were allowed a recovery period of two days before the first recording session began. On recovery, animals showed completely normal behavior, with no signs of distress. Animals were habituated to sit quietly during recordings which were made in the same recording chamber as that used for anesthetized animals. The stability of the head-holder enabled us to perform multiple recording sessions on each animal over a period of 4–6 weeks. To minimize animal stress, each recording session was limited to 3–5 h. Between recording sessions, animals showed no sign of stress or impairment due to the head anchor.

2.2. Recordings and microiontophoresis

Microiontophoretic administration of drugs was performed with an IONOPHOR 3 iontophoresis unit (Science Products) via three-barrel or five-barrel micropipettes (tip diameter 9–18 μm and 15–30 μm). Micropipette barrels were filled with a combination of the following solutions: 3 M-NaCl for extracellular recording and for automatic current compensation, BIC (bicuculline methiodide 10 mM, pH 3.0), gabazine (SR95331; 3 mM, pH 3.0), and GABA (0.5 M; pH 3.0). All drugs were obtained from Sigma. Ejecting currents ranged from 15 nA to 80 nA, and the retaining current was always −15 nA. Under microscopic control, the micropipette assembly was stereotaxically inserted through a small dural puncture and lowered into AI using a motorized stepping microdrive. Sites for pipette penetrations (spaced about 100–200 μm apart) were chosen in order to evenly sample the cortical surface whilst avoiding injury to blood vessels. Penetrations were made tangential to the cortical surface such that the pipettes remained for long distances in the middle layers of AI. Unit activity was recorded using a multi-channel recording system with 20,000× amplification, band-pass filtering (250 Hz 2-pole low cut-off and 8 kHz 6-pole high cut-off), and 40 kHz sampling at 12-bit resolution per recording channel (Multichannel Acquisition Processor, Plexon). In general, multiunit activity was recorded. In rare cases ($n = 4$), spike waveforms of single units were separated online using a spike sorting (template matching) algorithm (Sort Client, Plexon), which theoretically allowed up to 4 waveforms to be distinguished from multiunit recordings. Data from different spike clusters were then stored separately for offline analysis.

2.3. Acoustic stimulation

Acoustic stimuli (pure tones and AM tones) were delivered free field via an attenuator (PA4, Tucker Davies), an amplifier (STAX SRM-3) and an electrostatic speaker (STAX Lambda Nova). The speaker was mounted approximately 2 cm in front of the animal's head. Speaker out-

put was measured prior to an experiment using a 1/2-inch condenser microphone (Brüel & Kjær model 4133) placed at the position of the animal's head and facing the speaker. The signal from the amplifier was amplified (Brüel & Kjær model 2610), and monitored on a signal analyzer (Brüel & Kjær model 2033). For frequencies between 0.3 and 20 kHz the output of the speaker was found to be flat within ±5 dB and without distortion up to 90 dB sound pressure level (SPL). Stimuli were produced using a computer-controlled multifunction generator (DD1, System 2, Tucker Davies). AM signals of 100% modulation depth were generated by adding three sine waves, viz. the carrier frequency (fc) and two sidebands with half the amplitude of fc (fc+ modulation frequency and fc− modulation frequency). All components started at phase zero at stimulus onset. All stimuli were presented at a constant intensity of 65 ± 5dB SPL and had a duration of 200 (pure tones) or 500 ms (AM tones), with 5 ms rise and fall times. Neuronal activity was also recorded during a 50 ms pre-stimulus and a 150 ms post-stimulus period. Inter-stimulus intervals used were 0.5 s for pure tones and 0.75 s for AM tones.

2.4. Experimental procedure

Unit activity was recorded in the primary auditory cortex field AI. Verification of recording site was based on the known functional organization of the auditory cortex of the Mongolian gerbil (tonotopic gradients, temporal response characteristics of units like latency and temporal response pattern) as documented in the literature (cf. Thomas et al., 1993; Schulze et al., 1997). For each unit, the frequency response range (FRR) and the pure tone frequency evoking maximum response (best frequency; BF) were first determined approximately by presenting pure tones ranging from 0 to 20,000 Hz in 1000 Hz steps. For quantification of the effects of BIC and gabazine on frequency tuning, the range of pure tones presented was reduced to optimally cover the frequency response range of each unit. The BF thus determined served as the fc for subsequent AM tone stimulation. To test the pharmacological effectiveness of BIC and gabazine and to ensure that an adequate ejecting current was used for each drug, we first applied GABA with a current which was sufficient to inhibit a neuron's response to its BF and then ejected BIC or gabazine with a current which antagonized the GABA-induced inhibition of the acoustically evoked response. This ejecting current (which typically ranged from 15 nA to 40 nA; maximum: 80 nA) was then used to study the effects of BIC and gabazine on responses to pure and AM tones. Responses to pure tones or AM tones were recorded before (pre-drug), during (drug) and after (recovery) continuous iontophoresis of either BIC or gabazine. Pure tones of different frequencies and AM tones of different modulation frequencies (typically between 0 (=unmodulated carrier) and 30 Hz in 2 Hz steps) were presented in random order. Each complete set of pure or AM tones was presented 15 times, and stimuli were randomized separately for each presentation. Drugs were typically applied for between 6 and 14 min which was the time required for the presentation of one complete set of pure tones or one set of pure tones followed by one set of AM tones. However, in some cases longer durations of iontophoresis were used to study the dose-dependency of drug-induced effects. Following termination of drug application, stimuli were presented repeatedly (for up to ~45 min) until response magnitude and selectivity returned to pre-drug levels or the units were lost.

2.5. Data analysis

A neuron's BF was determined from rate functions, and its FRR was derived from PSTHs, after subtraction of spontaneous activity (which was averaged over all pre-stimulus periods for a given stimulus set). BF was defined as the pure tone frequency that evoked the highest discharge rate, and FRR as the range of frequencies that elicited an excitatory response. A response was regarded as significant if it was three SD above spontaneous activity. To obtain response rates at BF that were independent of response duration, response rates for all conditions tested in a given unit (pre-drug, drug, recovery) were determined using constant time windows. As the most tonic responses were usually obtained during the drug condition, analysis time windows were fitted to this condition comprising the complete excitatory response, and then applied to all other conditions. The FRR served as an estimate of the isointensity frequency receptive field. Sharpness of tuning of the isointensity responses was quantified by dividing the BF (as determined in the pre-drug condition) by the width of the FRR (Q-value). Response latency and response duration were determined from PSTHs with 2 ms resolution.

To quantify the degree of envelope synchronization (phase-locking) of neuronal discharges to AM tones, temporal modulation transfer functions (tMTFs; see insets to Figs. 2 and 4D–F) were generated by plotting vector strength (VS) as a function of modulation frequency. These were calculated from the responses to AM tones with modulation frequencies ranging from 0 Hz (unmodulated carrier) to 30 Hz in a time window omitting the ON-response. The significance of VS values was tested using Raleigh statistics (Mardia, 1972). VS values that were not significant at the $P < 0.05$ level were set to zero.

All experiments were conducted in accordance with the NIH Guidelines for Animals in Research and with the ethical principles defined by the German Law for the protection of experimental animals. The experimental protocols were approved by an ethics committee of the state of Sachsen-Anhalt, Germany.

3. Results

This study is based on analyses of recordings from a total of 86 units in AI for which complete data sets were obtained. The results obtained from anesthetized and unanesthetized animals were qualitatively very similar and will therefore be described together.

3.1. Effects of BIC and gabazine on pure tone responses

In response to pure tones, cells in AI of gerbils and other mammals typically show phasic discharges that are tuned to a certain range of pure tone frequencies (Thomas et al., 1993; Schulze et al., 1997). Fig. 1 shows representative examples of the effects of BIC (A–C) and gabazine (D–F) on the responses to pure tones in two different cells. In the pre-drug condition, the cell in Fig. 1A–C showed band-pass tuning ($Q = 0.8$) for a narrow range of pure-tone frequencies (1–3.5 kHz), with a BF of around 2 kHz. Application of BIC (Fig. 1B) resulted in substantial broadening of frequency tuning (FFR: 0.5–8.5 kHz; $Q = 0.25$) which reflected an increase in response to non-optimal frequencies both above and below the BF. Note also that the cell now responded well to a wide range of frequencies which were ineffective in the pre-drug condition (compare points above horizontal lines in insets to Fig. 1A and B). These new responses were of longer latency than the response to the BF. They are therefore reminiscent of the long-latency responses to non-optimal frequencies seen in most cells in the absence of drug application (Heil, 2004; cf. convex appearance of pure tone onset latencies in Fig. 1A, 4A and 5A. Latencies longer than the latency of the response to BF are most obvious for frequencies lower than BF). As was typically the case, broadening of frequency tuning during BIC application was accompanied by a marked increase in spontaneous activity (from 4.1 to 16.0 spikes/s), in the response rate at BF (from 24.0 to 119.5 spikes/s; compare insets to Fig. 1A and B) and in

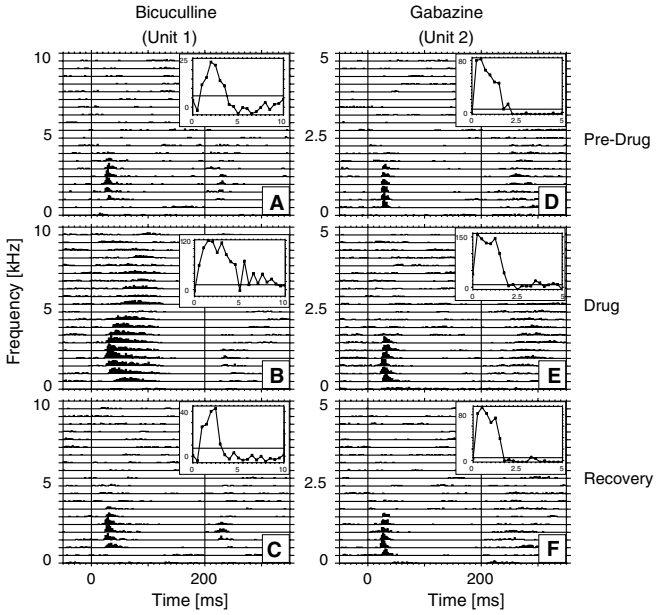

Fig. 1. Comparison of the effects of BIC and gabazine on responses to pure tones in two cells with similar pre-drug response characteristics (A–C and D–F) recorded in AI of the anesthetized gerbil. Each panel (A–F) shows a set of PSTHs in response to a range of pure tone frequencies (0–10 kHz in A–C and 0–5 kHz in D–F) before (pre-drug; A, D), during (drug; B, E) and after (recovery; C, F) iontophoretic application of BIC (left column) or gabazine (right column). Height of individual PSTHs within each panel (vertical calibration): A: 11, B and E: 16; C: 14; and D and F: 13 spikes/bin; vertical lines indicate onset and offset of stimulation; binwidth 2 ms, throughout. Ejecting current: B: 80 nA; E: 20 nA. Data in B,E and C,F derived 0–6 min after the onset and offset of drug application, respectively. Insets show FRRs for the on-responses in the corresponding PSTHs (responses to stimulus presentation; 0–200 ms), with spike rate (impulses/s) plotted as a function of pure tone frequency (in kHz); 0 = spontaneous activity; horizontal lines represent the criterion for significant responses.

the duration of the response at BF (from 26.3 ms to 125.3 ms). Interestingly, in this case the effects elicited by BIC on response magnitude and selectivity were observed only for the on-responses (during stimulus presentation) and not for responses to the offset of stimulation. In contrast to the dramatic BIC-induced effects on response selectivity for pure tones, application of gabazine had essentially no influence on frequency tuning (Q-value in both D and E: 0.2), despite the fact that it resulted in a marked increase in both spontaneous activity (from 2.8 to 6.6 spikes/s) and in the magnitude of the on-response at BF (from 82.0 to 158.2 spikes/s). Although in this case, application of gabazine had no significant effect on the duration of the response at BF (pre-drug: 18.0 ms; drug: 20.2 ms), it caused a substantial increase in response duration in many other cells (see Fig. 3). As is documented in Fig. 1C and F, all drug-induced effects were reversible.

3.2. Effects of BIC and gabazine on responses to AM tones

In response to AM tones, cells in AI typically show discharges which are phase-locked to the modulation frequency of the stimulus, that is, the temporal structure of the sound is encoded in the temporal structure of the neuronal discharges. Cells in AI of the gerbil can show phase-locked responses to AM tones modulated at frequencies of up to about 100 Hz (Schulze and Langner, 1997), although in most units phase-locking is confined to frequencies below 30 Hz. Fig. 2 compares the effect of iontophoretic application of BIC (A–C) and gabazine (D–F) on this type of phase-locked behavior in two different cells. In the pre-drug condition (A and D), phase-locked response components are evident as multiple peaks in the individual PSTHs whose occurrence rates vary with modulation frequency and which are locked to the phase of the modulation frequency. These response components can be distinguished from onset responses (the initial peak in the PSTHs) which are locked to stimulus onset with a latency which is independent of modulation frequency. As is evident from the tMTFs in the insets, which plot VS as a function of modulation frequency, both cells showed significant phase-locking (indicated by dots above data points) throughout the range of modulation frequencies tested up to 30 Hz. These phase-locked responses were differentially affected by the application of BIC and

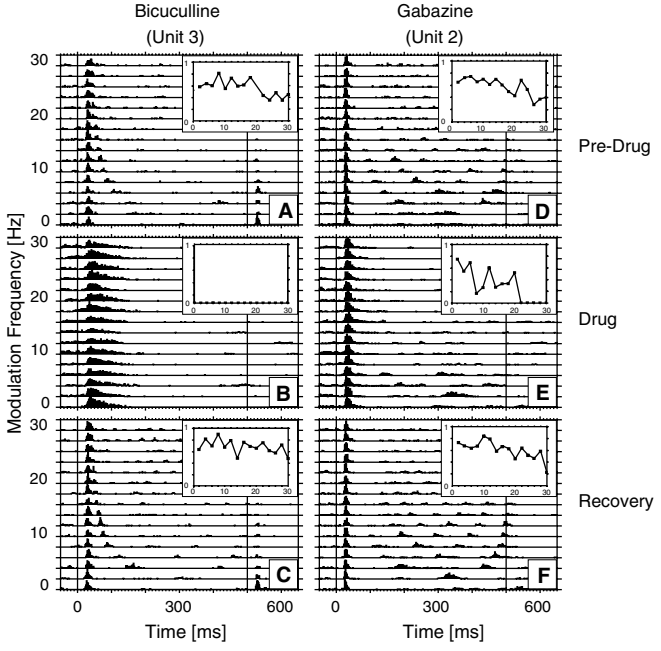

Fig. 2. Comparison of the effects of BIC and gabazine on responses to AM tones in two cells (A–C and D–F) recorded in AI of the anesthetized gerbil. Each panel (A–F) shows PSTHs in response to AM tones with a modulation frequency of 0–30 Hz, before (A, D), during (B, E), and after (C, F) iontophoresis of BIC (left column) or gabazine (right column). Vertical calibration of PSTHs: A: 15, B and F: 18, C: 17, D: 16, and E: 20 spikes/bin; binwidth 2 ms, throughout. Ejecting current: 80 nA. Data in B,E and C,F derived 0–8 min after the onset and offset of drug application. Insets show tMTFs (derived from the corresponding PSTHs), where VS is plotted as a function of the modulation frequency (in Hz) of the AM tone. VS values that were not significant at the $P < 0.05$ level have been set to zero. Dots above data points indicate significant phase locking.

gabazine. Whilst application of BIC (Fig. 2B) essentially eliminated phase-locked responses at all modulation frequencies tested, significant phase-locked responses to modulation frequencies up to 20 Hz were still present during application of gabazine (Fig. 2E). The abolition of phase-locked responses to higher modulation frequencies during gabazine application reflected the drug-induced increase in the duration of each phase-locked response component. This effectively reduced the highest modulation frequency that could be followed by the neuron's discharges in a phase-locked manner, because the duration of the response to each cycle of the modulation must be shorter than one cycle for phase-locking to be possible. Although application of BIC and gabazine elicited differential effects on phase-locked responses, both drugs induced a marked increase in spontaneous and stimulus-evoked activity. As is documented in Fig. 2C and F, all drug-induced effects were reversible.

3.3. Quantitative analysis of the effects of BIC and gabazine

A quantitative analysis of the effects elicited by BIC and gabazine application on responses to pure and AM tones is provided in Fig. 3 and in Tables 1 and 2. In the pre-drug condition, cells in the BIC group ($n = 56$) and the gabazine group ($n = 30$) did not differ significantly ($P > 0.05$; analysis of variance) in either mean sharpness of frequency tuning for pure tones ($Q = 0.73$ and 0.60) or mean upper cut-off frequency of envelope synchronization to AM tones (19.2 and 19.1).

Overall, application of both drugs resulted in a marked increase in the magnitude and duration of responses to BF for pure tones and in spontaneous firing rate (Fig. 3A–C and Table 1). As is documented in Table 1, all of these effects were highly statistically significant. They were somewhat greater in strength for BIC than for gabazine application and, in the case of spontaneous rate and response duration, this difference reached statistical significance (see Table 2). Application of both drugs also led to a slight increase (of ~1 ms) in the latency of response to BF (Fig. 3D and Table 1), although this effect reached statistical significance only for BIC application. The most striking difference between the effects elicited by BIC and gabazine application was seen in the sharpness of frequency tuning for pure tones. Overall, application of BIC resulted in substantial broadening of frequency tuning (decrease in Q) and this effect was highly statistically significant; by contrast, application of gabazine left frequency tuning essentially unchanged (Fig. 3E and Table 1). The difference in the magnitude of the effects elicited by BIC and gabazine was statistically significant (Table 2). It is important to note, however, that many cells did not show substantial broadening of frequency tuning during the application of BIC (Fig. 3E; and see below). Finally, application of BIC and gabazine also elicited differential effects on phase-locked responses to AM tones. Although application of both drugs resulted in a statistically significant decrease in the highest modulation frequency to which phase-locked responses could be elicited, the effect was much more pronounced for BIC application (Fig. 3E and Table 1). During the application of BIC, the upper cut-off modulation frequency was much lower than during the application of gabazine, and in several cases, phase-locked responses were abolished throughout the entire range of modulation frequencies tested. As can be seen in Table 2, the difference between the effects elicited by BIC and gabazine on phase-locked responses to AM tones was statistically significant.

3.4. Dose-dependence of drug-induced effects

The finding that iontophoresis of BIC could have deleterious effects on frequency tuning for pure tones and phase-locked responses to AM tones which were not observed during application of the more potent, selective $GABA_A$-antagonist gabazine suggested that these BIC-induced effects were due not to a blockade of GABAergic inhibition, but to the well-documented secondary effects of the drug described in the introduction. These secondary effects occur at higher drug concentrations than those required to block inhibitory synapses. We therefore surmised that the failure of BIC to substantially affect frequency tuning and phase-locking in some cells (see above) simply reflected a lower extracellular concentration and distribution of the drug (Hicks, 1984), and that in these cases effects might yet be elicited with prolonged drug application and high ejecting currents. Similarly, the absence of effects on frequency tuning and phase-locking during gabazine application might be argued to merely reflect limited diffusion of the drug to the inhibitory synapses controlling these response properties. These considerations motivated us to study in a subset of cells the effects of prolonged application of BIC or gabazine with increasing ejecting currents.

Examples of these experiments are shown in Figs. 4 and 5. Fig. 4 compares in the same cell the effect of BIC application on responses to pure and AM tones. Prior to BIC application, the cell showed band-pass tuning for pure tones, with a FRR of 0.6–2.6 kHz (Fig. 4A), and phase-locked responses to AM tones for modulation frequencies up to 30 Hz (Fig. 4D). Iontophoresis of BIC with a low ejecting current (Fig. 4B) resulted in an increase in spontaneous activity (from 5.1 to 12.9 spikes/s), and in the magnitude of the response to BF (from 43.0 to 110.1 spikes/s; compare insets to A and B). However, sharpness of frequency tuning for pure tones remained essentially unchanged (Q in A and B: 0.80 and 0.73). Additionally, whilst the BIC-application effectively abolished phase-locked responses to AM tones for the upper range of modulation frequencies tested, significant phase-locking was still observed for low modulation frequencies up to 12 Hz. These initial effects of BIC application are reminiscent of the influence of gabazine on responses to pure tones and AM tones (cf. Figs. 1, 2 and 5). In contrast, prolonged application of BIC with a higher ejecting current (Fig. 4C

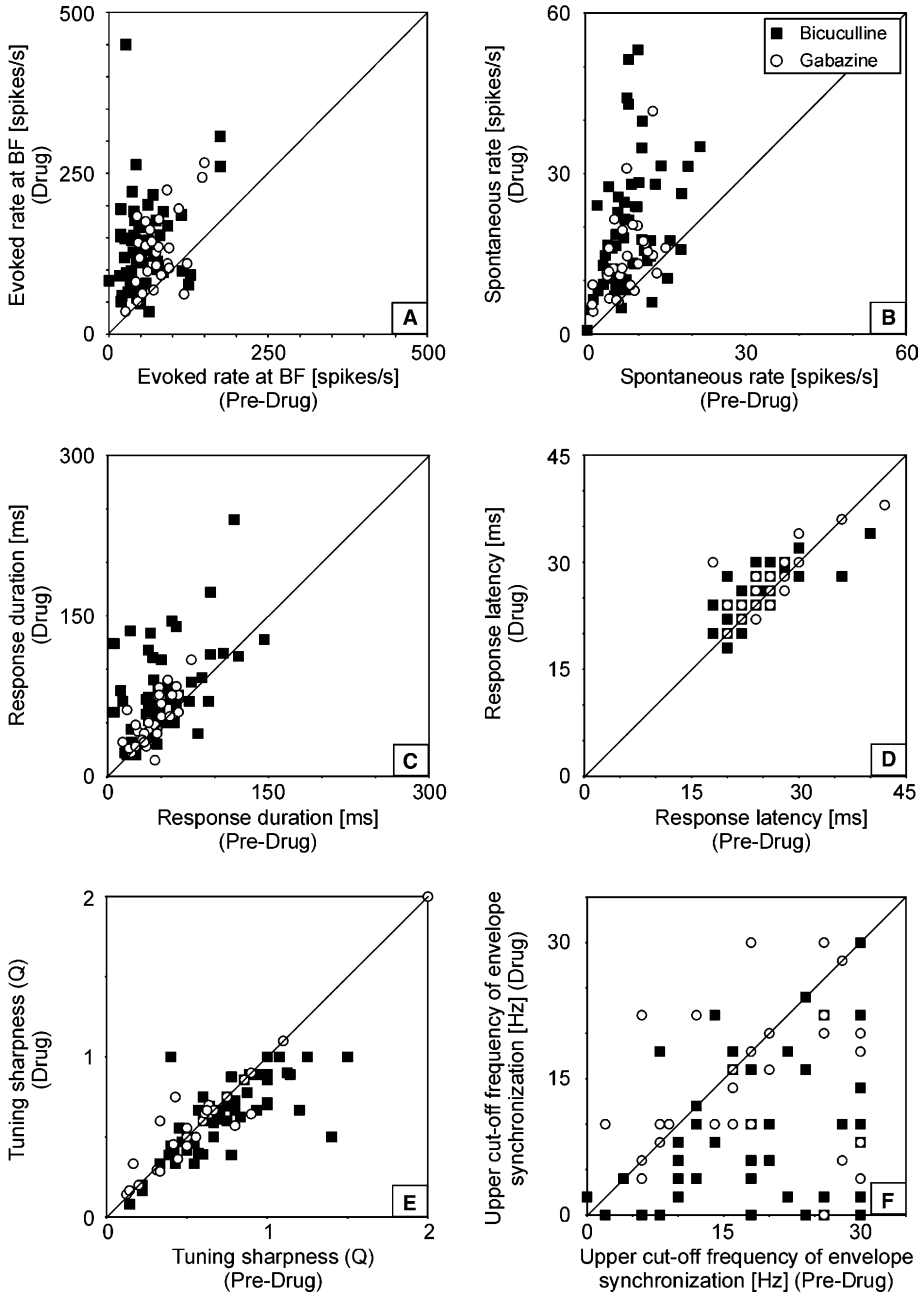

Fig. 3. Scatter diagrams of response parameters. Plotted are evoked and spontaneous rate (A and B), response duration and latency (C and D), sharpness of tuning for pure-tone frequency (E), and upper cut-off frequency of envelope synchronization to AM tones (F) prior to drug application (abscissa) against the same values obtained during drug application (ordinate). Diagonal lines indicate no change in the parameter plotted. Data points above and below this diagonal represent increases and decreases in a given parameter. Data for BIC and gabazine application are indicated by filled squares and open circles, respectively. Data from anesthetized and unanesthetized animals have been pooled. Data from experiments with continued application of BIC or gabazine over a long time period with increasing ejecting currents (cf. Figs. 4–6) are not included.

and F) had deleterious effects on both frequency tuning and phase-locked responses. The cell now showed substantial broadening of frequency tuning (Fig. 4C; decrease in Q from 0.80 to 0.44) which was due primarily to a marked increase in the response to frequencies above the cell's BF (1.6 kHz). Indeed, the cell now gave a vigorous response to a wide range of frequencies which were ineffective prior to drug application, and some of these responses (to 2.8 kHz and 3.2 kHz) exceeded the response to the original BF. In addition to eliciting dramatic effects on pure-tone responses, prolonged BIC application completely abolished phase-locked responses to the modulation frequency of AM tones (Fig. 4F). A complete recovery from all the BIC-induced effects occurred within approximately 30 min of the termination of iontophoresis (not shown). Dose-dependent effects similar to those documented in Fig. 4 were seen in all 15 cells which were subjected to prolonged application of BIC.

Table 1
Quantitative analysis of the effects of BIC and gabazine on responses to pure tones and AM tones

	Evoked rate at BF (spikes/s)			Spontaneous rate (spikes/s)			Response duration (ms)			Response latency (ms)			Tuning sharpness (Q)			Upper cut-off frequency of envelope synchronization (Hz)			n units	N animals
	Mean (SD)			Mean (SD)			Mean (SD)			Mean (SD)			Mean (SD)			Mean (SD)				
	Pre-drug	Drug	P	Pre-drug	Drug	P	Pre-drug	Drug	P	Pre-drug	Drug	P	Pre-drug	Drug	P	Pre-drug	Drug	P		
Bicuculline	56.8 (36.0)	138.6 (71.1)	$7.20E^{-12}$	8.5 (4.6)	19.6 (11.7)	$1.59E^{-10}$	50.6 (30.2)	76.0 (42.9)	$1.88E^{-6}$	23.6 (4.2)	24.6 (3.6)	0.002	0.73 (0.29)	0.64 (0.23)	0.0003	19.2 (8.3)	9.5 (7.7)	$7.63E^{-11}$	56	12
Gabazine	75.2 (30.7)	132.5 (55.3)	$2.09E^{-7}$	7.6 (3.7)	14.4 (7.7)	$3.82E^{-6}$	41.6 (16.3)	51.6 (22.9)	0.0006	25.7 (4.7)	26.4 (4.2)	0.10	0.60 (0.36)	0.61 (0.35)	0.32	19.1 (9.0)	14.3 (8.0)	0.01	30	10

The mean values shown in the "pre-drug" and "drug" columns were averaged over all cells in which the parameters at the top of the column were determined prior to and during the application of BIC or gabazine. Values in parentheses are the standard deviations of these mean values. Statistical significance of differences between means is indicated by the P values which were derived using the paired t-test. Additionally, non-parametric testing (resampling statistics, cf. Good, 2000) yielded qualitatively identical results (not shown). N = number of animals; n = number of units. Data from anesthetized and unanaesthetized animals have been pooled. Data from experiments with continued application of BIC or gabazine over a long time period with increasing ejecting currents (cf. Figs. 4–6) are not included.

Table 2
Quantitative comparison of the magnitude of BIC- and gabazine-induced changes in responses to pure tones and AM tones

	Evoked rate at BF (spikes/s)			Spontaneous rate (spikes/s)			Response duration (ms)			Response latency (ms)			Tuning sharpness (Q)			Upper cut-off frequency of envelope synchronization (Hz)		
	Mean (SD)			Mean (SD)			Mean (SD)			Mean (SD)			Mean (SD)			Mean (SD)		
	BIC	Gabazine	P	BIC	Gabazine	P	BIC	Gabazine	P	BIC	Gabazine	P	BIC	Gabazine	P	BIC	Gabazine	P
Drug-induced change	81.7 (72.1)	57.2 (48.3)	0.1	11.2 (10.9)	6.7 (6.8)	0.05	25.4 (36.7)	10.0 (15.2)	0.03	1.1 (2.7)	0.7 (2.8)	0.51	−0.10 (0.20)	0.01 (0.11)	0.008	−9.8 (9.2)	−4.8 (11.2)	0.03

The numbers shown in the "BIC" and "gabazine" columns are the drug-minus-pre-drug values for the parameter shown at the top of the column. Values are means averaged over all cells tested, with standard deviations of these means in parentheses. Statistical significance of differences between means is indicated by the P values which were derived using the analysis of variance. Although some of the data tested here were not normally distributed (as evaluated by the Lilliefors-test) we used the analysis of variance as it was demonstrated that this test is robust against violation of the normal distribution of the data (Box, 1954). Furthermore, we additionally tested all data that were not normally distributed with a resampling statistic (Good, 2000). This test gave qualitatively identical results as the analysis of variance in these cases (not shown). Data from anesthetized and unanaesthetized animals have been pooled. Data from experiments with continued application of BIC or gabazine over a long time period with increasing ejecting currents (cf. Figs. 4–6) are not included.

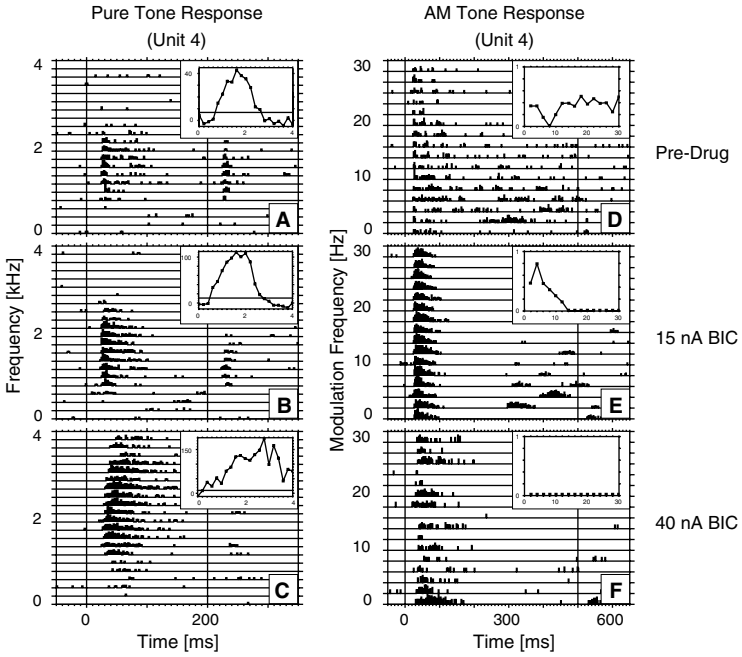

Fig. 4. Dose-dependent effects of BIC on responses to pure tones (A–C) and AM tones (D–F) in the same cell recorded from AI of the unanaesthetized gerbil. Conventions, derivation and layout as in Figs. 1 and 2. Vertical calibration of PSTHs: A: 8; B: 15; C: 13; D,F: 9; E: 17 spikes/bin; binwidth: 2 ms. Data in B and C derived 0–6 and 14–20 min after the onset of BIC iontophoresis; those in E and F derived 6–14 and 20–28 min after the onset of drug application. Ejecting current in B and E: 15 nA; in C and F: 40 nA.

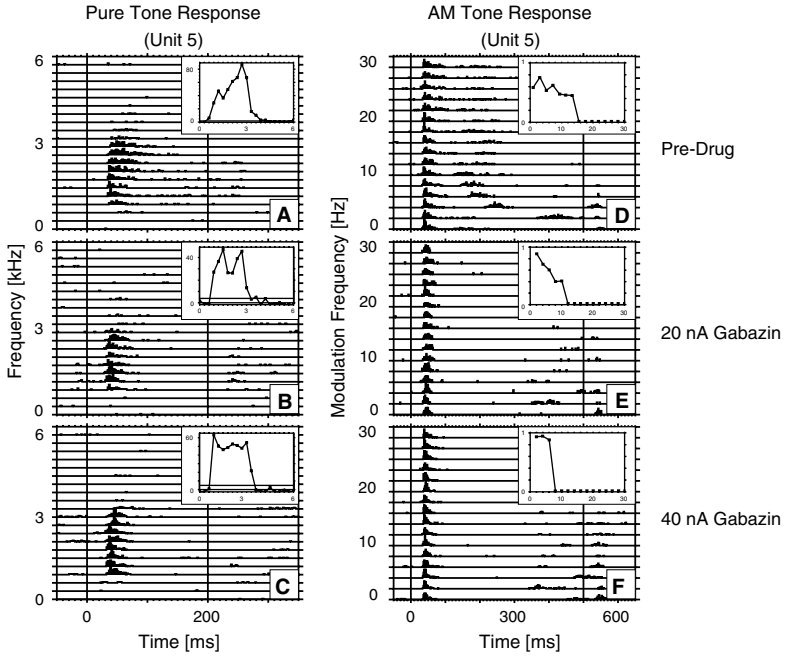

Fig. 5. Dose-dependent effects of gabazine on responses to pure tones (A–C) and AM tones (D–F) in the same cell recorded from AI of the unanaesthetized gerbil. Conventions, derivation and layout as in Fig. 4. Vertical calibration of PSTHs: A: 10; B: 9; C: 12; D–F: 14 spikes/bin; binwidth: 2 ms. Data in B and C derived 0–6 and 14–20 min after the onset of BIC iontophoresis; those in E and F derived 6–14 and 20–28 min after the onset of drug application. Ejecting current in B and E: 20 nA; in C and F: 40 nA.

Comparable experiments were performed with gabazine in 6 cells. Even with long periods of iontophoresis (up to 1 h) and high ejection currents (up to 80 nA), we did not observe the type of dose-dependent effects which were induced by prolonged BIC application. Fig. 5 compares in the same cell the effect of iontophoretic application of gabazine with increasing ejecting currents on responses to pure and AM tones. It is apparent that the cell's sharpness

of frequency tuning for pure tones remained essentially unchanged from its pre-drug value throughout the period of drug application (Q-values in A, B, C: 0.7, 0.8 and 0.7). Additionally, although in this cell the upper cut-off modulation frequency for AM tones decreased with increasing ejecting current (D: 14 Hz; E: 10 Hz; F: 6 Hz), phase-locked responses were by no means eliminated, as was the case during prolonged BIC application (Fig. 4F).

Fig. 6 provides a quantitative comparison of the dose-dependent influence of BIC and gabazine application on sharpness of frequency tuning for pure tones (top panel) and upper cut-off frequency of envelope synchronization to AM tones (bottom panel). Each datum point represents the average for all cells tested with different drug ejection currents. Relative to the pre-drug condition, application of BIC induced a significant reduction in both sharpness of frequency tuning and upper cut-off frequency of envelope synchronization at all ejection currents (paired t-test; $P < 0.01$). However, there was a dramatic increase in the magnitude of the effects of BIC application with increasing ejecting current (and duration of application). By contrast, application of gabazine did not induce a significant change in either parameter at any ejecting current ($P > 0.1$).

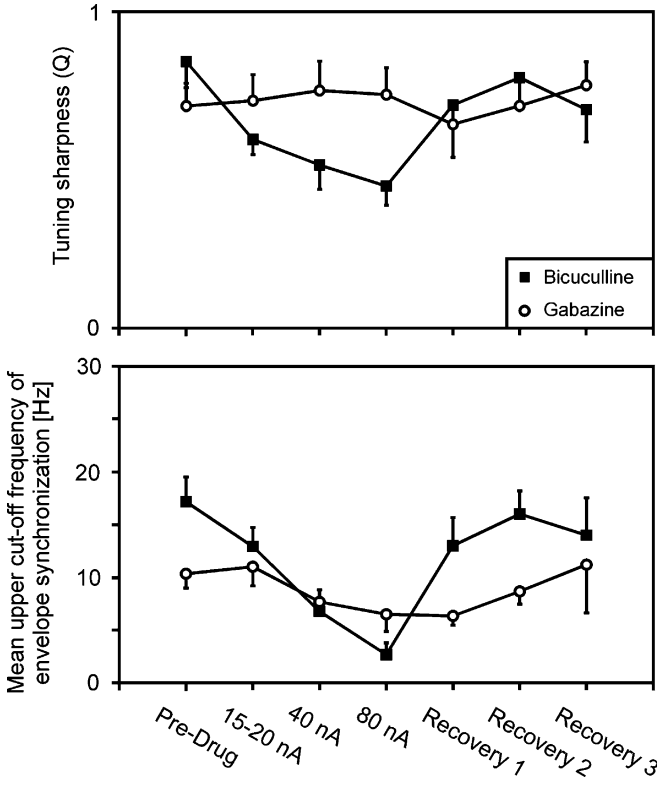

Fig. 6. Quantitative comparison of the dose-dependent effects of BIC and gabazine application on sharpness of frequency tuning for pure tones (top panel) and upper cut-off frequency of envelope synchronization to AM tones (bottom panel). Plotted are the mean values for all cells tested with different ejecting currents (indicated below). Short vertical lines are standard errors of these mean responses (drawn in one direction only).

4. Discussion

In this study, we have made the first direct comparison of the effects of in vivo iontophoretic application of two $GABA_A$-antagonists BIC and gabazine in sensory cortex. We have shown that in gerbil AI, iontophoresis of BIC and gabazine can elicit differential effects on response selectivity for pure and AM tones. Application of BIC often resulted in broadening of frequency tuning for pure tones and an elimination of phase-locked responses to AM tones, whereas application of gabazine left frequency tuning essentially unchanged and abolished phase-locked responses only to high modulation frequencies. Typically, short durations of BIC iontophoresis with low ejecting currents resulted in effects which were reminiscent of those elicited by gabazine application, whereas prolonged application with a higher ejecting current additionally resulted in broadening of frequency tuning and an elimination of phase-locked responses. The effects observed during iontophoresis of gabazine can be attributed to the loss of $GABA_A$-mediated inhibition. The effects which were elicited only during the application of BIC were presumably due to the well-documented side-effects of BIC which occur at high doses and include an action of the drug on calcium-dependent potassium channels (Olsen et al., 1976; Heyer et al., 1981; Johnson and Seutin, 1997; Johansson et al., 2001; cf. below). By using an acoustic stimulation paradigm which included both pure and AM tones, and by comparing the effects elicited by BIC and gabazine on the response selectivity to these stimuli, we have been able to (i) define the contribution of $GABA_A$-mediated inhibition to the spectral and temporal processing of sounds in AI; and (ii) demonstrate for the first time that iontophoretic application of BIC can have effects on neuronal response selectivity which are not due to blockade of GABAergic inhibition.

For the following reasons, the differential effects elicited by BIC and gabazine on frequency tuning and phase-locked responses cannot be attributed to differential diffusion of the two $GABA_A$-antagonists or to an inability of gabazine to block the relevant GABAergic synapses: (i) BIC and gabazine have been shown to have identical passive diffusion radii within AI (Richter et al., 1999); (ii) application of gabazine failed to influence frequency tuning for pure tones or eliminate phase-locked responses to AM tones even when the drug was applied for extended periods with high ejecting currents; (iii) the currents used for ejecting gabazine were always sufficient to antagonize the inhibitory action of iontophoretically applied GABA; and (iv) application of gabazine typically caused a marked increase in evoked and spontaneous discharge rate, indicating that it was reaching inhibitory synapses affecting the recorded cell.

A remaining question concerns the mechanisms underlying the broadening of frequency receptive fields we observed with high doses of BIC application. It is known from several studies that measured neuronal activity intra-

cellularly or cell-attached in vivo that the spectral width of the inputs reaching auditory cortical neurons is wider than the so-called classical receptive fields that are determined from spiking activity, that is, subthreshold spectral receptive fields are wider than suprathreshold spectral receptive fields (e.g., Ojima and Murakami, 2002; Wehr and Zador, 2003; cf. Metherate et al., 2005). It is has been suggested that such wide subthreshold or 'silent' inputs may play a role in certain mechanisms of neuronal plasticity (e.g., Rajan et al., 1993). We postulate that the action of BIC onto calcium-dependent potassium channels leads to a disturbance of repolarizing potassium currents that results in a sustained depolarization of the neurons. Consequently, under these circumstances, weak excitatory inputs that are subthreshold under control conditions could now be sufficient to elicit suprathreshold depolarization, i.e., action potentials as measured extracellularly in our study. Furthermore, the long latencies to non-optimal stimuli we observed in our study during the application of BIC in high doses are compatible with the long latencies reported for such subthreshold, off-BF inputs: It was previously shown that differences in latencies of onset membrane depolarizations could differ by up to about 70 ms between responses to BF and those to remote frequencies (cf. Ojima and Murakami, 2002; Fig. 2). To test whether the observed broadening of frequency receptive fields with high doses of BIC can indeed be explained by this proposed mechanism, we suggest a study in which the microiontophoretic application of BIC in different doses as we did it is combined with intracellular or cell-attached recording from auditory cortical neurons in vivo.

The results of the present study resolve the controversy about the potential contribution of $GABA_A$-mediated inhibition to frequency tuning for pure tones in AI. In AI, iontophoretic application of BIC has been reported to result in substantial broadening of frequency tuning (chinchilla AI: Wang et al., 2000, 2002; gerbil AI: Foeller et al., 2001), and similar results have been reported for the auditory cortex analogue of the chick forebrain (Müller and Scheich, 1988). These results were interpreted as being due to a blockade of $GABA_A$-mediated inhibition which normally sharpens frequency tuning by suppressing responses to non-optimal frequencies. By contrast, Schulze and Langner (1999) found that iontophoresis of BIC in gerbil AI had essentially no influence on frequency tuning for pure tones, and they concluded that $GABA_A$-mediated inhibition does not contribute to frequency selectivity in AI. Our results from gerbil AI confirm this interpretation, for we found that iontophoresis of gabazine (a far more potent $GABA_A$-antagonist than BIC) left frequency tuning for pure tones essentially unchanged. The absence of an inhibitory mechanism to sharpen frequency tuning within AI makes functional sense. The cells providing the input to AI are already sharply tuned for pure-tone frequency by virtue of lateral inhibitory mechanisms operating at lower levels of the auditory pathway (Rhode and Greenberg, 1994; Yang et al., 1992). In the light of the present results, the BIC-induced expansion of frequency receptive fields reported by Wang, Foeller and co-workers can be attributed mainly to the secondary effects of BIC application rather than reflecting a loss of GABAergic inhibition. Consistent with this interpretation, Dykes et al. (1984) reported that the receptive fields of cells in cat somatosensory cortex could be enlarged by the application of BIC, but were unaffected by the application of GABA. The BIC-induced changes in receptive field size were thus presumably mediated by non-GABAergic mechanisms.

Although the present study has provided strong evidence that GABAergic inhibition does not contribute to spectral tuning at the level of AI, it has demonstrated the importance of $GABA_A$-mediated inhibitory mechanisms for temporal processing in this area. Iontophoresis of both BIC and gabazine often resulted in a marked reduction in the highest modulation frequency of AM tones to which cells showed phase-locked responses. In other words, neurons lost their ability to faithfully represent in their firing patterns the fast temporal modulations of sound envelopes. These effects were typically accompanied by an increase in the duration of evoked responses, suggesting that $GABA_A$-mediated inhibition serves both to enhance the temporal resolution of responses to pure tones and to preserve in neuronal responses the temporal features of complex sounds. Although iontophoresis of BIC often eliminated phase-locked responses to all modulation frequencies tested, this effect could not be replicated by the application of gabazine. During gabazine application, most cells showed significant phase-locked responses to a wide range of low modulation frequencies. This implies that the influence of $GABA_A$-mediated inhibition on temporal processing is limited to fast temporal modulations of sound envelopes. The fast inhibitory postsynaptic potentials mediated by $GABA_A$-receptors would certainly make them ideally suited to this task.

Iontophoresis of BIC has been used extensively at multiple levels of different sensory systems in an attempt to elucidate the functional role of $GABA_A$-mediated inhibition. A critique of these studies is well beyond the scope of the present paper. However, studies which have been performed in visual cortex have yielded particularly controversial results. In cat visual cortex, iontophoresis of BIC has been shown to disrupt neuronal selectivity for the orientation and direction of motion of contours (Sillito, 1975b; for review see Sillito, 1984; Eysel et al., 1998), implying that these properties are sharpened by intracortical inhibition, rather than being established via a convergent excitatory input from the lateral geniculate nucleus (Hubel and Wiesel, 1962). Similarly, in macaque primary visual cortex (Sato et al., 1995) and middle temporal area (Thiele et al., 2004), iontophoretic application of BIC has deleterious effects on direction selectivity, suggesting that the generation of this property in these areas depends heavily on intrinsic inhibitory circuitry. On the other hand, in cat visual cortex, some studies have reported only modest effects of BIC application on cortical orientation and direc-

tion selectivity (Vidyasagar and Heide, 1986; Albus and Baumfalk, 1988), whilst intracellular studies (reviewed in Ferster (1994)) have found little evidence for a major contribution of intracortical inhibition to either property. Pertinently, the most dramatic effects of BIC application on cortical orientation/direction selectivity have often been observed during prolonged application of the drug with high ejecting currents. In the light of the present results, it is likely that some of these effects were due to non-GABAergic secondary effects of BIC application rather than or in addition to the blockade of $GABA_A$-receptors. Although there is independent pharmacological evidence for a contribution of inhibition to cortical orientation/direction selectivity (Crook and Eysel, 1992; Crook et al., 1998; reviewed in Crook et al. (2002)), the effects observed during iontophoretic application of BIC should not be taken to unequivocally reflect the precise contribution made by $GABA_A$-mediated inhibitory processes to these properties.

It has been known for more than 20 years that, in addition to blocking $GABA_A$-receptors, BIC has several non-GABAergic side-effects which may influence neuronal activity in a dose-dependent manner. Here, we have shown for the first time that iontophoretic application of BIC in vivo can have deleterious effects on neuronal response selectivity which are not due to blockade of $GABA_A$-mediated inhibition. Consistent with the dose dependency of the different effects of BIC application in vitro, these non-GABAergic effects typically occurred during prolonged drug application with high ejecting currents. However, they could also be observed within 6–8 min of the onset of drug application (Figs. 1A–C and 2A–C) and with ejecting currents as low as 15 nA. This presumably reflects the lack of a straightforward relationship between ejected current and extracellular drug concentration in vivo (Hicks, 1984). The secondary effects that can be elicited with high doses of BIC application combined with the difficulty of controlling drug concentration using microiontophoresis limits the usefulness of iontophoretic application of BIC for assessing the role of $GABA_A$-mediated inhibition. For this purpose, the more potent $GABA_A$-antagonist gabazine, which does not seem to induce the non-GABAergic side-effects associated with the application of BIC (Chambon et al., 1985; Heaulme et al., 1986; Hamann et al., 1988), would seem to be the drug of choice. In the light of the present work, studies in which iontophoresis of BIC has been used in an attempt to elucidate the functional role of $GABA_A$-mediated inhibitory processes need to be re-evaluated.

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