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ACTIONS OF BICUCULLINE ON CELL BODY AND NEUROPILAR MEMBRANES OF IDENTIFIED INSECT NEURONES

S. D. BUCKINGHAM[1], B. HUE[2] AND D. B. SATTELLE[1,*]

[1]*AFRC Laboratory of Molecular Signalling, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK* and [2]*Laboratoire de Neurophysiologie CNRS URA 611, Faculté de Médecine, Université d'Angers, Rue Haute de Reculée, 49045 Angers, France*

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Summary

Bicuculline and its methochloride salt block inhibitory postsynaptic potentials recorded from giant interneurone 2 (GI 2) of the cockroach *Periplaneta americana* following stimulation of cercal nerve X, but fail to block the response to γ-aminobutyric acid (GABA) when this neurotransmitter is ionophoresed or pressure-injected onto the fine branches of GI 2 within the neuropile of the terminal abdominal ganglion. Bicuculline is similarly ineffective in blocking the response recorded when GABA is ionophoresed onto the cell body membrane of GI 2.

The cell body membranes of cockroach GI 2 and fast coxal depressor motor neurones have been used to show that bicuculline blocks cell body (extrasynaptic) neuronal nicotinic acetylcholine receptors. Pressure-injection of acetylcholine into the neuropile results in a depolarization of GI 2 that is blocked by bicuculline. Therefore, the block by bicuculline of inhibitory postsynaptic potentials recorded from GI 2 may result in part from actions at sites other than synaptic GABA receptors. Alternatively, there may exist a population of synaptic GABA receptors on GI 2 that, unlike GI 2 cell body GABA receptors, are sensitive to bicuculline.

Introduction

Several laboratories have studied the actions of bicuculline, a plant alkaloid that acts as a specific $GABA_A$ receptor antagonist in vertebrates, on GABA receptors of insects. Bicuculline blocks inhibitory postsynaptic potentials (IPSPs) recorded from interneurones of the moth (*Manduca sexta*) antennal lobe (Hildebrand, 1988; Waldrop *et al.* 1987). In contrast, bicuculline insensitivity is reported for GABA-mediated IPSPs recorded from an identified locust (*Schistocerca gregaria*) interneurone (Watson and Burrows, 1987). Studies of IPSPs recorded from the cell body (extrasynaptic) membrane

*To whom correspondence should be addressed.

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of the fast coxal depressor motor neurone (D_f) of the cockroach *Periplaneta americana* show that these are not blocked by bicuculline at resting membrane potential (Sattelle *et al.* 1988). Similar findings are reported for extrasynaptic GABA receptors on unidentified, locust (*Locusta migratoria*) neuronal cell bodies (Benson, 1988) and embryonic, cultured cockroach (*Periplaneta americana*) neurones (Beadle and Lees, 1985). To date, the actions of bicuculline on responses to GABA of both neuropile and cell body membranes of a uniquely identified insect neurone have not been tested.

The nervous system of the cockroach (*Periplaneta americana*) has been used for characterization of insect GABA receptors by means of radioligand binding, ligand-induced anion uptake and electrophysiology (Sattelle, 1990; Anthony *et al.* 1993). Radioligand binding experiments on cockroach nerve cords (Lummis and Sattelle, 1985, 1986), together with those performed on locust head ganglia (Breer and Heilgenberg, 1985; Lunt *et al.* 1985) and housefly heads (Abalis and Eldefrawi, 1986), have identified a putative bicuculline-insensitive GABA receptor. Bicuculline insensitivity is also observed when receptors are assayed using GABA-induced uptake of $^{36}Cl^-$ into membrane microsacs prepared from the cockroach nervous system (Wafford *et al.* 1987). Electrophysiological studies on motor neurone D_f reveal a bicuculline-insensitive GABA receptor that is blocked by picrotoxin, and for which the following order of effectiveness of agonists has been demonstrated: muscimol > GABA > 3-aminopropanesulphonic acid (3-APS) (Sattelle *et al.* 1988). Earlier studies on unidentified cockroach cell bodies described neuronal responses to GABA that exhibit sensitivity to bicuculline (Pitman and Kerkut, 1970; Walker *et al.* 1971). In our own initial studies, certain batches of bicuculline did produce suppression of the insect neuronal GABA response (David and Sattelle, 1984), but this was never seen with the methiodide salt and has not been observed in experiments in which cells were tested at their normal resting membrane potential. In a few experiments, partial suppression of the GABA response was seen at hyperpolarized membrane potentials (Sattelle *et al.* 1988). Recently, bicuculline has been found to block both *in situ* (Benson, 1988) and functionally expressed neuronal nicotinic acetylcholine receptors (Marshall *et al.* 1990).

In the present study we have therefore examined in detail the actions of bicuculline on the responses to GABA (and acetylcholine) of neuropile and cell body regions of an identified cockroach giant interneurone.

Materials and methods

All experiments were performed on adult male cockroaches (*Periplaneta americana*). For oil-gap recordings from giant interneurone 2 (GI 2), animals were dissected dorsally and the abdominal nerve cord removed. The sixth abdominal ganglion and one connective between the sixth and fifth abdominal ganglia were desheathed using two sharpened insect pins. The desheathed connective was dissected by teasing away all other axons leaving the axon of GI 2 intact (Pichon and Callec, 1970). The preparation was then transferred to an oil-gap recording chamber (Callec, 1974). Potentials were stored on a Histomat computer for further analysis. Compounds were applied to the giant

interneurone by pressure-injection or by ionophoresis. By applying drugs into the neuropile or at the surface of the ganglion they could be delivered locally to neuropile or cell body (extrasynaptic) membranes of GI 2. Glass micropipettes filled with 1.0 mol l^{-1} GABA or 0.1 mol l^{-1} acetylcholine were controlled by a Neurophore micropressure injection system. Bicuculline was ejected from the pipette by 10–30 ms pulses of pressure of 140–200 kPa delivered at a frequency of 50–100 Hz. GABA and acetylcholine were ejected as a single pulse of pressure of variable amplitude and duration. The dose of agonist was varied by altering either duration or pressure of the pulse.

Intracellular recordings were made from the cell body of GI 2 and the cell body of the fast coxal depressor (D_f) motor neurone, as described in detail elsewhere (Harrow and Sattelle, 1983). Animals were dissected dorsally and either all of the abdominal nerve cord (for experiments on GI 2), or the mesothoracic, metathoracic and first abdominal ganglia (in the case of experiments on motor neurone D_f), were excised. The ganglionic sheath was removed from the ventral surface of the ganglion using two pairs of sharpened forceps and the preparation was then transferred to an experimental chamber. In both types of experiment, acute angles of illumination allowed cell bodies to be observed. The distinctive size and position of their cell bodies facilitated the location of giant interneurone 2 and motor neurone D_f. The cell body was penetrated by a glass microelectrode, filled with 2.0 mol l^{-1} potassium acetate (electrode impedance 10–20 MΩ). All potentials were amplified on a WPI amplifier and displayed both on an oscilloscope and on a pen recorder. Bicuculline methochloride (Sigma Chemical Co., UK) and bicuculline methiodide (Pierce, UK) were diluted to the final concentration from a 0.1 mol l^{-1} stock solution.

Results

Effects of bicuculline on inhibitory post synaptic potentials recorded from giant interneurone 2

Giant interneurone 2 (GI 2) possesses synaptic receptors to GABA in the terminal abdominal ganglion, which mediate inhibitory postsynaptic potentials (IPSPs) (Callec, 1974). The oil-gap recording technique was used to determine the effects of 10^{-4} mol l^{-1} bicuculline on GABA-mediated IPSPs. Electrical stimulation of nerve X evoked an IPSP in GI 2 (see Bernard *et al.* 1983) which was completely and reversibly abolished by injection of 10^{-4} mol l^{-1} bicuculline into the neuropile (Fig. 1). In the same preparations, reversible block was also obtained in the presence of 10^{-8} mol l^{-1} picrotoxin. A complete block by bicuculline was achieved within 3 min of application, and a complete recovery was seen within 5 min of washout. Bicuculline, unlike picrotoxin, also abolished the EPSP produced by stimulation of nerve X.

These results do not show whether bicuculline acts directly on the GABA receptors that are located postsynaptically upon the cell membrane of GI 2 or on other components of the neural pathway. The different effects of bicuculline and picrotoxin on the evoked EPSP, however, suggest that bicuculline might act, at least in part, on sites other than the GABA receptor complex.

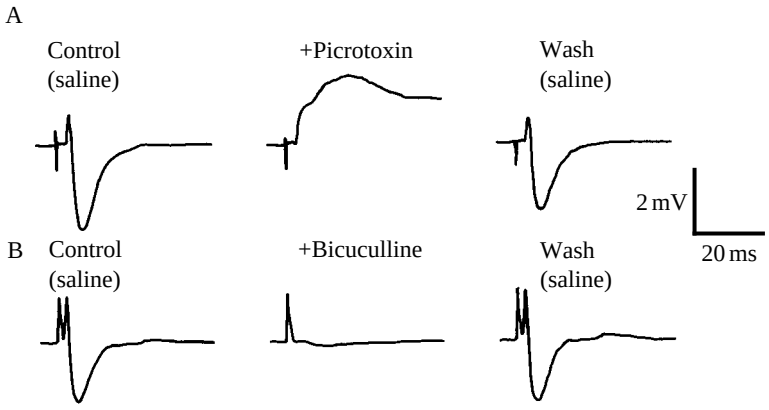

Fig. 1. Effects of (A) picrotoxin (10^{-8} mol l^{-1}) and (B) bicuculline (10^{-4} mol l^{-1}) on the compound IPSP evoked in giant interneurone 2 by electrical stimulation of cercal nerve X. Both compounds block the IPSP, but bicuculline, unlike picrotoxin, also blocks the small EPSP, leaving only the stimulus artefact.

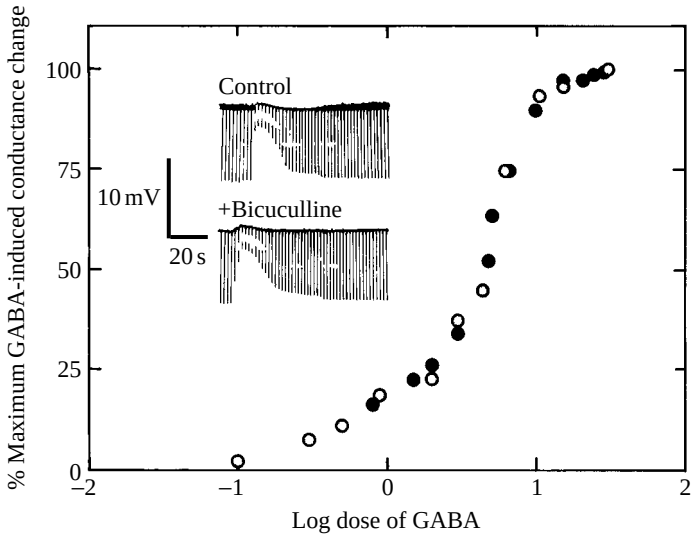

Fig. 2. The dose–response curve for the actions of GABA on GI 2 (●) is unaffected by the presence in the ganglion of 10^{-4} mol l^{-1} bicuculline (○). The log dose of GABA (in mol l^{-1}) is plotted against the resultant peak change in membrane conductance determined by potential changes induced by constant 2 nA current pulses delivered through the balanced recording electrode.

Effects of bicuculline upon responses of giant interneurone 2 to GABA applied by ionophoresis

Direct effects of bicuculline on GABA receptors on the dendrites of GI 2 were tested by ionophoretic application of GABA into the A6 ganglion neuropile in the presence and absence of 10^{-4} mol l^{-1} bicuculline simultaneously pressure-injected into the neuropile.

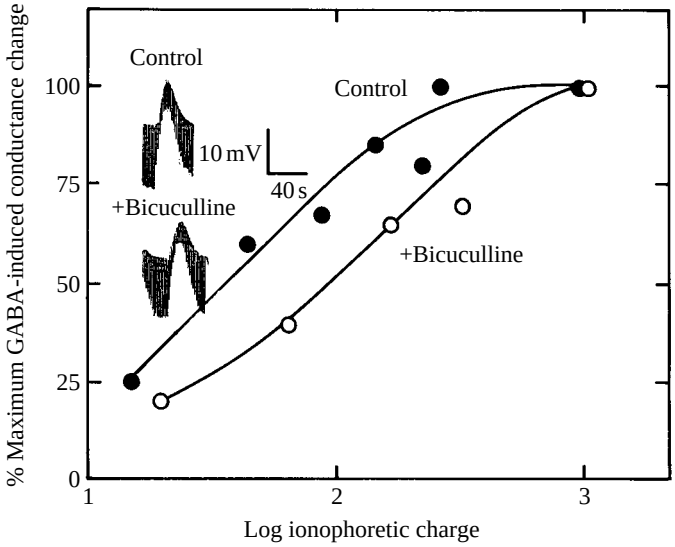

Fig. 3. Responses recorded in the cell body of GI 2 to GABA applied by ionophoresis near the cell body (●) are only slightly changed by a 20 min preincubation with 10^{-4} mol l^{-1} bicuculline (○).

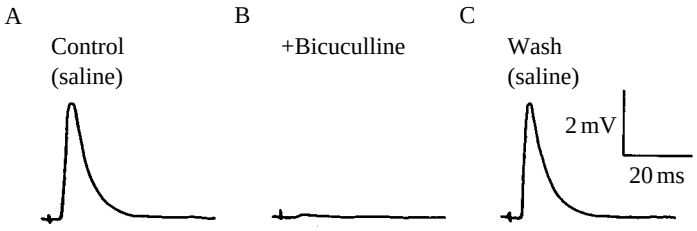

Fig. 4. The compound EPSP evoked by electrical stimulation of nerve XI (A) is reversibly reduced by pressure injection of 10^{-4} mol l^{-1} bicuculline into the ganglion (B,C).

Ionophoretic application of GABA into the neuropile resulted in a dose-dependent increase in membrane conductance that was unaffected by a simultaneous pressure-injection of 10^{-4} mol l^{-1} bicuculline into the neuropile (Fig. 2). Direct effects of GABA on extrasynaptic receptors located on the cell body of GI 2 were assayed by ionophoresing GABA through a micropipette positioned close to the cell body, whilst recording from the cell body with an intracellular microelectrode. GABA induced a dose-dependent increase in membrane conductance that was unaffected, or at most only slightly reduced by, a prior 20 min bath-application of 10^{-4} mol l^{-1} bicuculline (Fig. 3). This indicates that bicuculline has no effect on a population of GI 2 cell body and neuropile GABA receptors, and that the effects on the IPSPs evoked by nerve stimulation are attributable either (a) to actions on other elements of the neural pathway, or (b) to actions on a pharmacologically distinct population of synaptic GABA receptors that are bicuculline-sensitive.

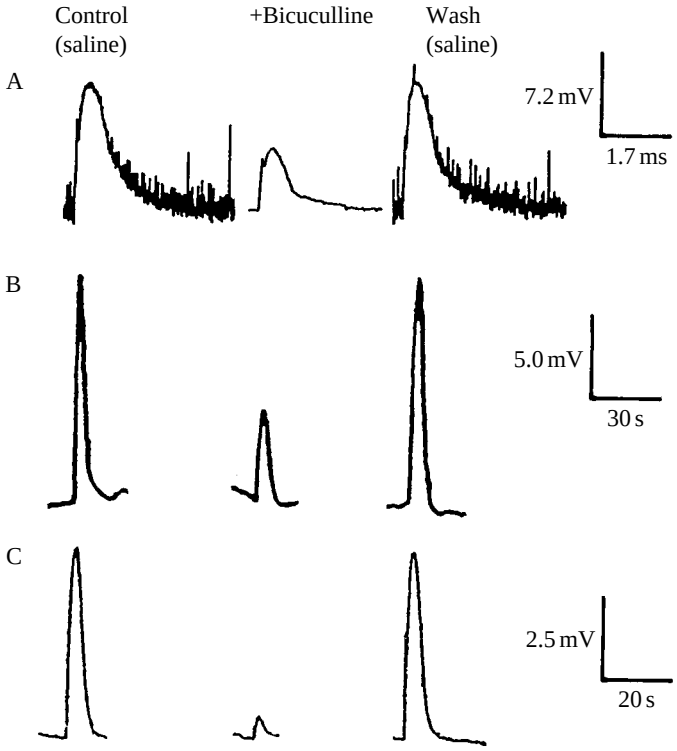

Fig. 5. (A) The response of GI 2 to acetylcholine pressure-injected into the region of its dendritic branches is reversibly reduced in the presence of 10^{-4} mol l^{-1} bicuculline. Spontaneous EPSPs are also reversibly abolished. (B) The response of GI 2 to 10^{-3} mol l^{-1} acetylcholine pressure-ejected onto the surface of the ganglion near its cell body is reversibly reduced in the presence of 10^{-4} mol l^{-1} bicuculline. (C) The response of motor neurone D_f to acetylcholine pressure-ejected in the region of its cell body is reversibly reduced in the presence of 10^{-4} mol l^{-1} bicuculline.

Effects of bicuculline on acetylcholine receptors

Electrical stimulation of nerve XI evokes a cholinergic EPSP in GI 2 (Callec, 1974). To test the possibility that bicuculline antagonizes acetylcholine action, we determined the effects of pressure-injected bicuculline on evoked EPSPs. Injection of 10^{-4} mol l^{-1} bicuculline into the neuropile of the A6 ganglion reversibly reduced the amplitude of EPSPs evoked by stimulation of nerve XI (Fig. 4). Pressure-injection of 10^{-3} mol l^{-1} acetylcholine into the neuropile resulted in a membrane depolarization that was reversibly reduced by a simultaneous injection of 10^{-4} mol l^{-1} bicuculline (Fig. 5A). Injection of the bicuculline salt also abolished the naturally occurring background EPSPs (Fig. 5A). This demonstrates that 10^{-4} mol l^{-1} bicuculline antagonizes the action of acetylcholine on synaptic receptors in the membrane of GI 2.

In addition to acetylcholine receptors in its neuropilar branches, GI 2 is also known to possess extrasynaptic acetylcholine receptors on its cell body (Harrow and Sattelle, 1983), which lies near the surface of the ganglion. To determine the effects of bicuculline on GI 2 cell body cholinergic receptors, acetylcholine (10^{-3} mol l^{-1}) and bicuculline

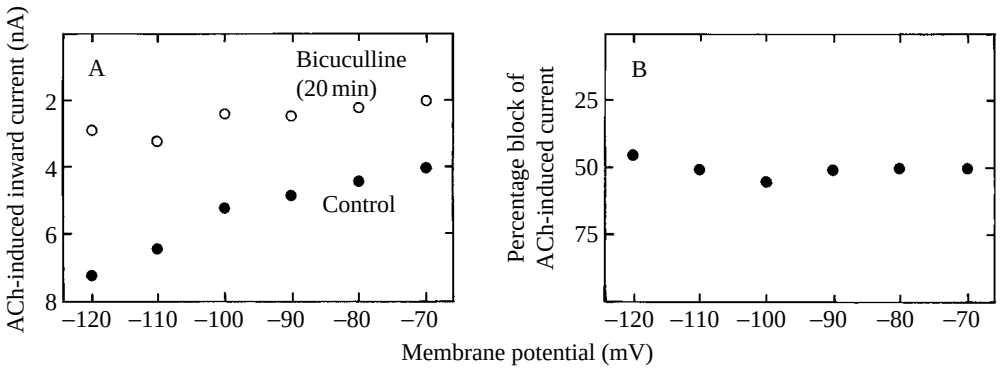

Fig. 6. (A) The relationship between the amplitude of the inward current evoked by ionophoretic application of acetylcholine (ACh) and the membrane potential at which the cell is clamped. The current–voltage relationship is shifted in the presence of 10^{-4} mol l^{-1} bicuculline. (B) The block is independent of membrane potential over the range −70 mV to −120 mV.

(10^{-4} mol l^{-1}) were pressure-injected individually, or in combination, from micropipettes located near the interneurone's cell body. Application of acetylcholine resulted in a membrane depolarization which was reversibly reduced by a simultaneous application of 10^{-4} mol l^{-1} bicuculline (Fig. 5B). Similar experiments were performed on the cell body of the identified fast coxal depressor (D_f) motor neurone, which is located near the ventral surface of the metathoracic ganglion. Pressure ejection of 1.0 mol l^{-1} acetylcholine (ACh) near the cell body resulted in a membrane depolarization that was reversibly reduced by a simultaneous ejection of 10^{-4} mol l^{-1} bicuculline (Fig. 5C). The amplitude of the current induced in the cell body by ACh was measured for a range of membrane potentials under voltage-clamp. Block of the response to ionophoretically applied acetylcholine by 10^{-4} mol l^{-1} bicuculline was independent of membrane potential over the range −70 mV to −120 mV (*N*=3) (Fig. 6).

Discussion

Walker *et al.* (1971) first detected a blocking action of bicuculline on cockroach acetylcholine receptors. Benson (1988, 1992) confirmed this finding for unidentified cultured locust neurones. The present report provides the first demonstration of antagonism by bicuculline of cholinergic receptors on identified insect neurones. This effect is observed on both neurite and cell body receptors of giant interneurone 2, and cell body receptors of motor neurone D_f. Therefore, bicuculline-sensitive acetylcholine receptors of the nicotinic type are present on neurones which differ widely in their functions. Cell body GABA receptors of motor neurone D_f and GI 2, and neurite GABA receptors of GI 2, are all insensitive to bicuculline.

Previous pharmacological studies on cholinergic receptors of the motor neurone D_f (David and Sattelle, 1984; Pinnock *et al.* 1988; Sattelle and David, 1983) have shown that

neuronal nicotinic receptors on this cell differ from both peripheral and central vertebrate cholinergic receptors. For example, this insect nicotinic receptor is blocked by both α-bungarotoxin (Sattelle, 1985, 1986) and κ-bungarotoxin (Chiappinelli *et al.* 1989; Pinnock *et al.* 1988), and is relatively insensitive to decamethonium (David and Sattelle, 1984). The demonstration that insect nicotinic cholinergic receptors are blocked by bicuculline provides further evidence that, like insect GABA receptors, they do not fit readily into the existing vertebrate classification of receptor subtypes, but form a pharmacologically distinct subtype of nicotinic receptor.

In contrast to its action on vertebrate $GABA_A$ receptors, bicuculline has no effect on dose-dependent, GABA-induced membrane conductance changes in giant interneurone 2 of the cockroach. This is in keeping with findings from several laboratories (see Sattelle, 1990) that bicuculline has no effect on the GABA response or on GABA binding in insect muscle or nervous tissue. The methiodide salt of GABA, which was used in some experiments, was found to be less potent, although we made no quantitative comparison of this with the methochloride salt. However, a recent report (Waldrop *et al.* 1987) has suggested that bicuculline antagonizes GABA-mediated IPSPs in unidentified interneurones in the moth, *Manduca sexta*. The finding that bicuculline may act on receptors other than those for GABA indicates that any apparent effect of bicuculline upon naturally evoked IPSPs might be mediated by another receptor type. The study by Waldrop *et al.* (1987) did not include a test of bicuculline action upon exogenously applied GABA, but the observed inhibition of synaptic IPSPs by bicuculline cannot be explained by blockade of presumptive nicotinic receptors elsewhere in the pathway, since the accompanying excitation, shown by Waldrop and Hildebrand (1988) to be mediated by acetylcholine receptors, is unaffected by bicuculline. The one direct test made for bicuculline action upon cholinergic responses to both nerve stimulation and application of acetylcholine (Waldrop and Hildebrand, 1988) revealed no action of bicuculline. Unfortunately for the present discussion, the single neurone upon which this test was performed responded both to acetylcholine and to nerve shock with a hyperpolarization, whereas the response to ACh in cockroach GI 2 and D_f, and most *Manduca* antennal lobe projection neurones, is depolarizing. Comparisons of data from several insect species indicate that there are subtypes of pharmacologically distinct GABA receptors in insects, some of which differ in their sensitivity to bicuculline (cf. Anthony *et al*. 1993).

The injection of ligands into the neuropile does not guarantee selective action on synaptic receptors. Waldrop *et al.* (1987) have shown that GABA injected into the neuropile of *Manduca* antennal lobe reduces and even abolishes IPSPs known to be mediated by GABAergic synapses. If this effect is attributed to desensitization of synaptic GABA receptors by the exogenous GABA, then it can be concluded that injected GABA has access to synaptic receptors. Furthermore, the reported block by bicuculline of cholinergic synaptic transmission (this study) demonstrates that this molecule also has access to synaptic receptors. If it is assumed, therefore, that the response observed in GI 2 following GABA injection into the neuropile contains a significant contribution from synaptic receptors, then the absence of any effect of bicuculline on the dose–response curve for GABA indicates a lack of action of bicuculline upon synaptic GABA receptors. However, because the proportion of the response mediated by synaptic neuropilar GABA

receptors, as opposed to extrasynaptic neuropilar GABA receptors, is unknown, our findings do not exclude the existence in this cockroach preparation of multiple GABA receptor types, only some of which are sensitive to bicuculline.

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