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SHORT COMMUNICATION

INHIBITORY EFFECT OF L-GLUTAMATE ON THE NEUROPILE ARBORIZATIONS OF FLIGHT MOTONEURONES IN LOCUSTS

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In vertebrates, L-glutamate (glutamate) is a major excitatory neurotransmitter in the brain, acting on at least three separate receptors (Fagg, 1985). In insects, there is strong evidence that glutamate is the excitatory neurotransmitter at the neuromuscular synapse (Usherwood, 1981). Since some motoneurones make central synapses (Watson and Burrows, 1982; Burrows *et al.* 1989), it is probable that glutamate is also released centrally. Indeed, Bicker *et al.* (1988) found glutamate-like immunoreactivity in the central projections of known glutamatergic motoneurones and in interneurones in the locust, and Watson (1988) has shown it to be localized at central synapses. Furthermore, isolated insect neuronal somata respond to glutamate by activation of a chloride conductance (Usherwood *et al.* 1980), and ionophoretic applications of glutamate in certain areas of the neuropile cause motoneurones to depolarize and to spike (Sombati and Hoyle, 1984). In cultured embryonic cockroach brain neurones, Horseman *et al.* (1988) found both excitatory and inhibitory responses to glutamate. Wafford and Sattelle (1989) found only hyperpolarizing responses to glutamate on the fast coxal depressor motoneurone soma *in situ*, but they recorded depolarizing responses to kainate and quisqualate. These experiments indicate that receptors for glutamate are present on insect neuronal somata. However, since insect neuronal somata play no role in synaptic transmission and are generally not electrically excitable (Gwilliam and Burrows, 1980), the function of somal neurotransmitter receptors is unclear. To study the role of putative neurotransmitters in mediating or modulating normal synaptic inputs it is necessary to apply the substances to the neuropile where the synapses are and to record the effects there. It is also necessary to use an animal preparation where normal synaptic inputs can be recorded. In this study, the effects of topical pressure applications of glutamate were monitored by means of intracellular recordings, in the neuropile, from flight motoneurones (FMNs) during fictive flight behaviour. While this procedure does not ensure that only synaptic receptors are activated, it does enable one to activate synaptic receptors or study the effect that extrasynaptic receptors have on synaptic activity.

Key words: neurotransmitter, L-glutamate, amino acid transmitters, insects, motoneurones, neuropharmacology.

Adult locusts (*Locusta migratoria*) were prepared as described previously (Robertson and Pearson, 1982). The wings and legs were removed, the dorsal cuticle opened and the internal organs removed. The exposed meso- and metathoracic ganglia were glued to a grounded metal plate and all nerves sectioned, except the two anterior connectives and N6 of the mesothoracic and N1 of the metathoracic ganglia. The body cavity was filled with Vaseline, leaving only the meso- and metathoracic ganglia exposed. After desheathing the metathoracic ganglion, the preparation was superfused with saline (in $mmol\,l^{-1}$: NaCl, 155; KCl, 3; $CaCl_2$, 4; Hepes, 10; sucrose, 25; pH adjusted to 6.8 with NaOH) at 5–10 $ml\,min^{-1}$. Fictive flight was elicited by directing air onto the head and was monitored extracellularly with electromyograms from muscle 112 (the dorsal longitudinal muscle, an indirect depressor) whose motoneurones in the meso- and metathoracic ganglia were left intact. Intracellular recordings were made in the neuropile of the metathoracic ganglia with thin-walled glass microelectrodes (resistance: 10–20 MΩ) filled with 3 $mol\,l^{-1}$ potassium acetate or 2.5 $mol\,l^{-1}$ potassium chloride. FMNs were identified by the dorsal location of their dendritic arborizations and their characteristic activity during flight episodes (Hedwig and Pearson, 1985). In a few cases, neurones were visualized by filling them with Lucifer Yellow (Stewart, 1981) to confirm their identity. Glutamate (sodium salt, Fluka; 10–100 $mmol\,l^{-1}$ in the pipette) was pressure applied on the desheathed surface of the ganglion from micropipettes positioned 10–50 μm from the intracellular electrode. The pressure ejection pipettes were made from 1–7 glass capillaries, glued together and pulled on a vertical Narishige puller. After filling, the common tip was 'bumped' back to a diameter of 2–10 μm and the assembly connected to a pressure valve system and checked for leakage. Pressure pulses (34.5–69 kPa) of 10–100 ms duration were triggered by a multichannel stimulator (A.M.P.I.). Solutions of various ionic composition were either superfused or pressure ejected. The results reported below are based on over 150 preparations.

Upon impalement, the FMNs showed a resting potential (RP) between −50 and −70 mV. During the first minute, the RP usually stabilized at −60 to −75 mV. Intense synaptic activity was typical at rest, and during fictive flight the pattern of synaptic inputs was the same as that recorded from sheathed ganglia or that recorded with less extensive preparation procedures (Fig. 1A). Spiking threshold was usually 15–20 mV above RP.

Pressure applications of glutamate evoked two types of responses in the FMNs at RP (Fig. 1C). The most common response (46 % of the cells) was a 2–10 mV hyperpolarization (H response) lasting up to 60 s for larger applications (either longer pulses or higher pressure). These cells had a mean RP of -65.5 ± 5.5 mV (N=22). The H response was accompanied by a conductance increase, causing decreases of up to 50 % in the amplitude of the responses to negative current injection and drastic reduction of the amplitude of spontaneous synaptic potentials. The apparent reversal potential of the response, measured by injecting current through the recording electrode, was always more negative than RP (Fig. 2A). Because the FMNs have long and extremely ramified dendrites and thus

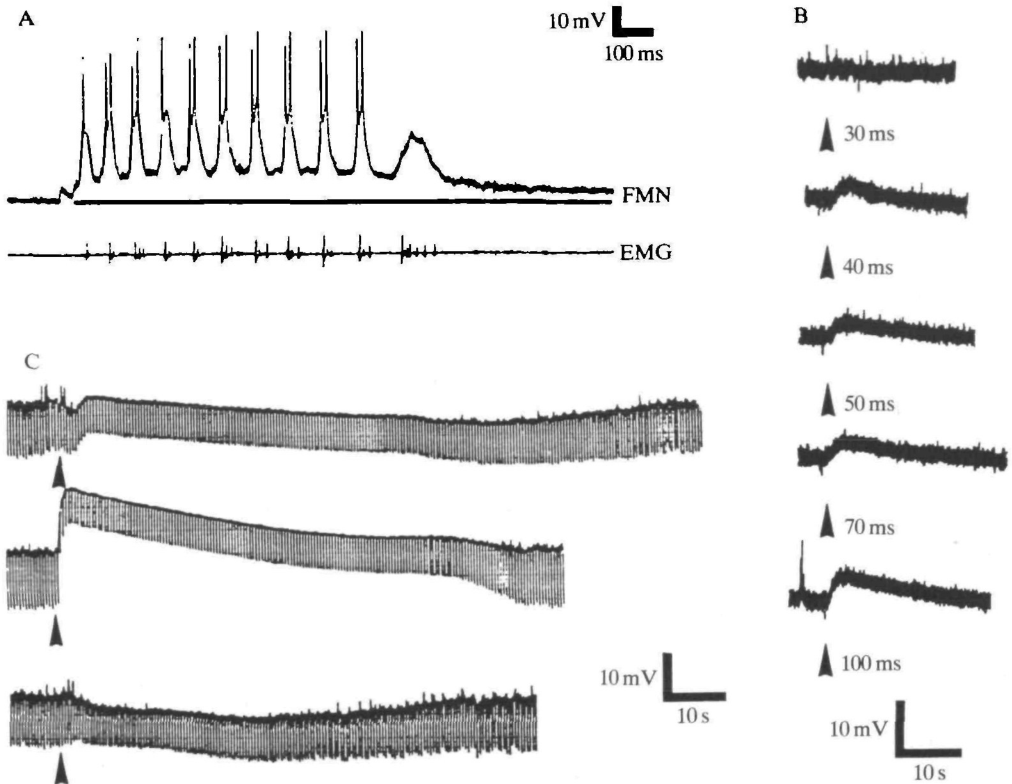

Fig. 1. (A) Flight motoneurone activity during fictive flight, recorded intracellularly from a metathoracic depressor motoneurone (FMN) and extracellularly from the depressor muscle 112 (EMG). (B) Dose-dependence of responses evoked by glutamate pulses (10 mmol l^{-1} in the pipette) of increasing duration (indicated below the trace). In this cell, a maximum depolarizing response was obtained with a 100 ms pulse. (C) Responses elicited in three FMNs of the same preparation by glutamate (100 mmol l^{-1} in the pipette; pulse duration 50 ms). Upper trace: biphasic response (B response) consisting of a depolarization superimposed on a hyperpolarization. Middle trace: depolarizing response (D response). Lower trace: hyperpolarizing response (H response). Constant-current hyperpolarizing pulses (100 ms, 0.5 nA) were applied to measure membrane resistance. The arrowheads mark the time of glutamate injection.

cannot be space-clamped, and because the action of glutamate could have taken place at a distance from the electrode, it was not possible to measure reversal potential values precisely. When the cells were electrically depolarized to their spiking threshold, glutamate application produced a large hyperpolarization and inhibited spiking.

In 38 % of the cells, glutamate application triggered a 2–5 mV depolarization (D response) but, even in these cells, spikes were never elicited (Fig. 1C). These cells had a more negative RP (-70 ± 6 mV, $N=10$). The D response was also accompanied by a conductance increase. The reversal potential of the D response was always more positive than RP (Fig. 2A). Thus, at spiking threshold, the D

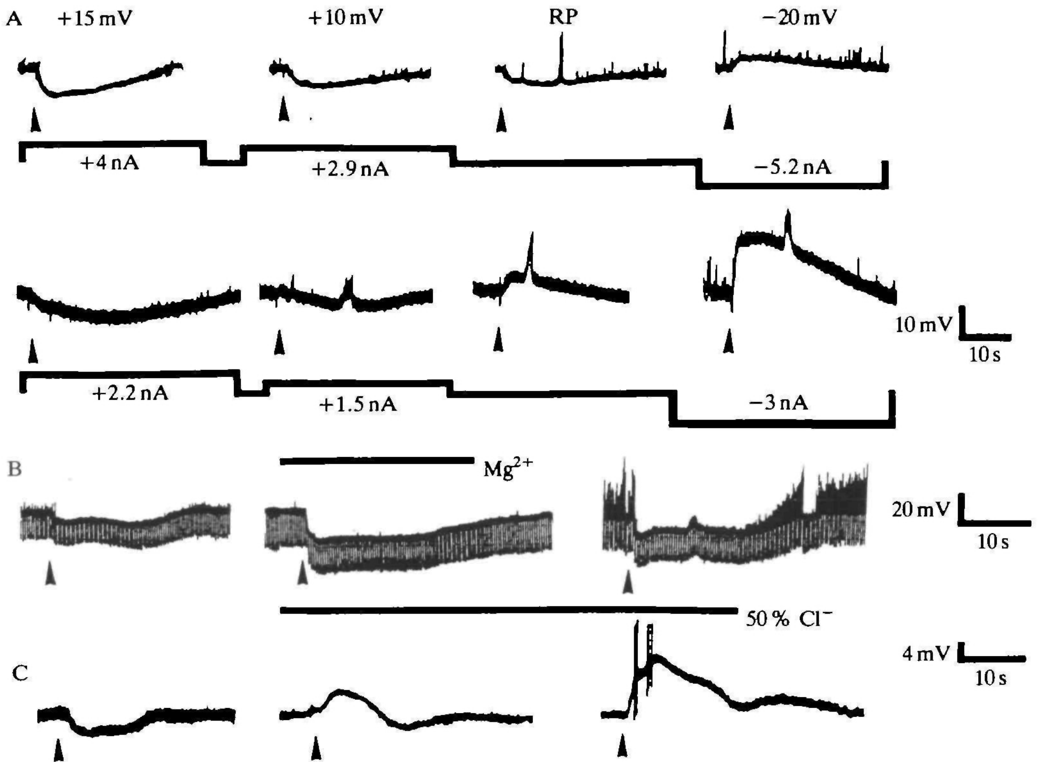

Fig. 2. (A) Reversal potential of H and D responses recorded in two different FMNs. The cell on the upper trace showed an H response at RP (−64 mV) which reversed at potentials below RP. In contrast, the cell on the lower trace produced a D response at RP (−72 mV) which reversed at depolarized potentials. Numbers indicate apparent voltage (mV) above or below RP at which both cells were held and, for each cell, the injected current (nA) necessary to achieve this shift. (B) Effect of addition of 20 mmol l^{-1} $MgCl_2$ in the extracellular solution. In the control solution, this cell responded with a hyperpolarization (left trace). Synaptic inputs were blocked within 2 min in the high-magnesium solution but the response to glutamate remained (middle trace). In this cell, the glutamate-evoked response was enhanced and remained so for several minutes after return to control solution (right trace). (C) Effect of lowering the extracellular chloride concentration. In the control solution (left trace), this cell also responded with a hyperpolarization. In 50 % chloride (substituted with gluconate), the response reversed (middle trace) and, after several minutes (right trace), the amplitude of the reversed response was sufficient to trigger spikes.

response, too, was hyperpolarizing and spontaneous spiking was inhibited. Even at spiking threshold, an increase in spiking frequency was never evoked in any FMN by any dose of glutamate, although in other cells of the same preparation glutamate could elicit spiking at RP.

The two types of responses were recorded in both elevator and depressor motoneurones and were present in different FMNs in the same preparation. Both

were dose-dependent (Fig. 1B) and were recorded with either potassium chloride or potassium acetate intracellular electrodes.

To test whether the glutamate effects on the FMN are direct, synaptic transmission was blocked by omitting $CaCl_2$ from the normal solution and adding $20\,mmol\,l^{-1}$ $MgCl_2$ (Fig. 2B). Spontaneous synaptic activity and inputs from the flight pattern generator were blocked within minutes in the impaled neurone, but the mesothoracic flight pattern generator remained functional since the electromyogram of muscle 112 (with motoneurones in the undesheathed mesothoracic ganglion) was normal. After blocking synaptic transmission, H and D responses were still present, suggesting that the responsible mechanisms were postsynaptic.

These data can be interpreted as evidence for a single glutamatergic increase in conductance, with the resulting membrane potential change depending on the relationship between membrane potential and the reversal potential of the conductance involved. This is supported by the observation that cells showing a D response had a more hyperpolarized RP than cells showing an H response.

In a few cells (16 %), the response to glutamate was biphasic (B response), consisting of a depolarization superimposed on a slower hyperpolarization (Fig. 1C). The two components of the B response differed in reversal potential: the hyperpolarizing component reversed at a potential more negative than RP, while the amplitude of the depolarizing component decreased with depolarization. The most likely explanation for this effect is that different parts of the cell, while having the same glutamate receptors, differ in their RP or have unhomogeneous extra- or intracellular ionic concentrations (Nicoll, 1988). Owing to the large space constant of the FMNs (Schneider and Kutsch, 1989), responses originating in different parts of the cell would be 'seen' at the recording site.

For somata, Usherwood *et al.* (1980) and Wafford and Sattelle (1989) have shown that the glutamate responses are chloride-dependent. In the FMNs, the apparent reversal potential of the D and H responses close to RP also suggests that they may be mediated by a chloride conductance. To test this, extracellular solutions with 75 or 50 % of the chloride concentration in the original solution were used (NaCl substituted by sodium gluconate). In low-chloride solution, H responses reversed, becoming D responses, and their amplitude was sufficient to reach spiking threshold (Fig. 2C). In contrast, the amplitude of D responses increased, as predicted by the Nernst equation.

During fictive flight activity, application of small doses of glutamate hyperpolarized the cell and reduced the amplitude of the synaptically driven, rhythmical oscillations in potential and the number of spikes per burst. Larger doses of glutamate hyperpolarized the cells more and reduced the amplitude of the synaptic potentials to below spiking threshold (Fig. 3). The flight pattern generator, however, was not affected by localized applications of glutamate, since synaptic potentials were still present and the electromyogram of muscle 112 was normal. These effects of glutamate on flight activity were recorded in FMNs showing either H or D responses at RP.

Taken together, these results suggest that, as in other invertebrate neurones

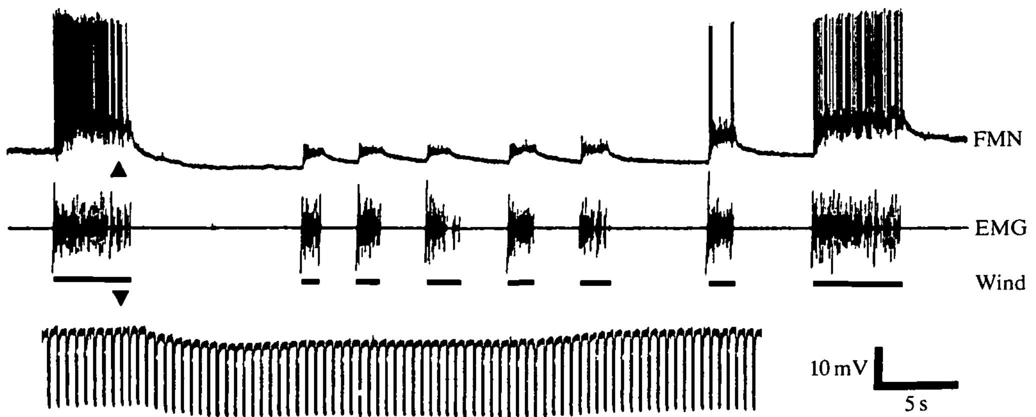

Fig. 3. Effect of glutamate application on FMN activity. Application of glutamate ($100\,\mathrm{mmol\,l^{-1}}$ in the pipette; pulse duration 100 ms) during fictive flight activity triggered by puffs of air initially reduces the amplitude of the rhythmical synaptic inputs and decreases the number of spikes per burst. During the course of the glutamate-evoked response, the amplitude of synaptic inputs elicited by subsequent puffs of air are reduced below spiking threshold. Upper trace: intracellular activity recorded from a FMN and caused by puffs of wind on the head. Middle trace: extracellular activity recorded from muscle 112. Lower trace: response evoked from the same cell and with the same dose of glutamate but in the absence of puffs of wind. Constant-current pulses were applied as in Fig. 1C.

(Kehoe, 1976; Marder and Paupardin-Tritsch, 1978; Walker *et al.* 1981), glutamate has an inhibitory effect on the FMNs and that this response is mediated, at least in part, by a chloride conductance. The inhibitory effect is depolarizing (D response) or hyperpolarizing (H response) at RP, depending on whether the membrane potential is above or below the chloride equilibrium. These findings also suggest that glutamate receptors mediating hyperpolarizations are present on the neuropile arborizations of locust FMNs, as found in isolated somata of locust metathoracic ganglia (Giles and Usherwood, 1985) and in cockroach neuronal somata in culture (Horseman *et al.* 1988) and *in vivo* (Wafford and Sattelle, 1989). This is the first time that inhibitory responses to glutamate have been recorded in the neuropile, an indication that, unlike somal receptors, they might play a role in either synaptic transmission or in the modulation of synaptic transmission.

The experiments presented here show that glutamate applications not only prevent spontaneous spiking at depolarized potential but also reduce the amplitude of synaptic inputs, for example from the flight pattern generator. It cannot be concluded, however, that the receptors mediating this action of glutamate are clustered at classical synapses. They could be distributed over the membrane of the neuropile arborizations like the extrasynaptic receptors mediating inhibitory responses in muscles (Cull-Candy, 1982). Reduction of synaptic inputs could be caused by a modulatory effect based on changes of membrane conductance and thus receptors distributed over the whole arborization could be very effective in preventing any inputs from reaching spiking threshold.

In the FMNs, evidence was found only for an inhibitory action of glutamate. This is in contrast with the results of Sombati and Hoyle (1984), who found that glutamate, released in the neuropile, excites leg flexor motoneurones. The difference does not seem to be due to the glutamate application system employed because it has been possible to bring other neurones (clearly not FMNs) to spiking threshold in the same preparations where glutamate had an inhibitory effect on the flight motoneurones. Thus, it seems likely that the excitatory receptors mediating the effect of glutamate on leg motoneurones are lacking in FMNs, although the presence of rapidly desensitizing excitatory glutamate receptors (Dudel *et al.* 1988) cannot be excluded.

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