GABAergic and Glutamatergic Inhibition of Nonspiking Local Interneurons in the Terminal Abdominal Ganglion of the Crayfish

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ABSTRACT
Nonspiking local interneurons in the terminal abdominal ganglion of the crayfish Procambarus clarkii receive inhibitory inputs from mainly glutamatergic spiking local interneurons and GABAergic nonspiking interneurons. In this study, the inhibitory responses of nonspiking interneurons to local application of glutamate and GABA into the neuropil were compared. Glutamate and GABA injection mediated the hyperpolarization of the nonspiking interneurons with an increase in membrane conductance. The glutamate-mediated membrane hyperpolarization was reversed by injection of 1 or 2 nA hyperpolarizing current. By contrast, more than 3 nA hyperpolarizing current was frequently necessary to reverse the GABA-mediated hyperpolarization. Bath application of a chloride channel blocker, 50 μM picrotoxin (PTX), reduced the glutamate-mediated hyperpolarization, but had no effect on the GABA-mediated hyperpolarization. The GABA-mediated hyperpolarization was not consistently affected by bath application of low chloride solution. These results suggest that the glutamate-mediated inhibition was related to the gating of a Cl⁻ conductance, while the GABA-mediated inhibition was not. Electrical stimulation of sensory afferents innervating the exopodite elicited ipsps in uropod opener motor neurons. These sensory-evoked ipsps were also PTX-insensitive, suggesting GABAergic nonspiking interneurons could be the predominant premotor elements in organizing the uropod motor control system. J. Exp. Zool. 303A:66–75, 2005. © 2005 Wiley-Liss, Inc.

INTRODUCTION
Inhibitory synaptic interactions play a crucial role in sensory processing and motor control. For example, lateral inhibition or reciprocal inhibition within sensory systems sharpens the directional sensitivity of sensory inflow. Furthermore, tonic inhibition and disinhibition of the motor system control the gain of motor outflow.

γ-aminobutyric acid (GABA) is the most widely distributed inhibitory neurotransmitter in both the vertebrates and invertebrates (e.g., Takeuchi and Takeuchi, '65). In the crustacean central nervous system, glutamate functions as an inhibitory transmitter (Marder and Paupardin-Tritsch, '78; Marder, '87). Some leg motor neurons in the crayfish have non-specific GABA/glutamate receptors (Pearlstein et al., '94), while swimmeret motor neurons have picrotoxin-insensitive GABA receptors and picrotoxin-sensitive glutamate receptors (Sherff and Mulloney, '96). The pharmacological profile of stomatogastric motor neurons to both GABA- and glutamate-mediated inhibition is variable (Marder and Paupardin-Tritsch, '78; Tazaki and Chiba, '94).

Nonspiking local interneurons in the terminal abdominal ganglion of the crayfish receive convergent peripheral and central inputs and control the activity of uropod motor neurons (Nagayama et al., '94). They receive inhibitory inputs via spiking and nonspiking local interneurons, while they receive excitatory inputs through sensory afferents and intersegmental interneurons (Nagayama, '97; Namba and Nagayama, 2004). Double labeling experiments using intracellular staining and immunocytochemical labeling

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show that many spiking local interneurons are glutamatergic (Nagayama et al., 2004), while nonspiking interneurons are mainly GABAergic (Nagayama et al., ’97). Thus, nonspiking interneurons are affected by both GABAergic and glutamatergic inhibition. In this study, the inhibitory responses of nonspiking interneurons to local application of GABA and glutamate within the neuropil are examined physiologically. The results show that a glutamate-mediated hyperpolarization of the nonspiking interneurons is picrotoxin-sensitive and linked to chloride conductance, while a GABA-mediated hyperpolarization of the nonspiking interneurons is picrotoxin-insensitive, and many would be potassium dependent. Furthermore, the inhibitory response of the uropod opener motor neurons to sensory stimulation is mediated by GABAergic synaptic transmission.

**MATERIALS AND METHODS**

Adult male and female crayfish, Procambarus clarkii Girard, of 7–9 cm body length from rostrum to telson, were used in all experiments. They were obtained commercially and kept in laboratory tanks with running fresh water, and fed weekly on a diet of chopped potato and liver.

The abdomen was isolated from the thorax and pinned ventral side-up in cooled van Harreveld’s (‘36) solution. The terminal (sixth) abdominal ganglion was exposed by removing the fifth sternite and peeling off the ventral aorta and connective tissue. The soft cuticle overlying uropod muscles was removed, along with the appropriate portions of the protopodite and exopodite, to monitor the activity of uropod motor neurons using suction electrode. The closer, reductor motor neuron was recorded extracellularly from the cut end of the nerve root 2 motor bundle at the bifurcation to the reductor and adductor exopodite muscles. The opener motor neurons were recorded from the nerve root 3 motor bundle at the bifurcation to the ventral rotator and the abductor exopodite muscles (Nagayama et al., 2002). Another suction electrode was placed over the cut end of the nerve root 2 sensory bundle to stimulate the sensory neurons innervating hairs on the surface of the exopodite. The terminal ganglion was stabilized on a silver platform and treated with protease (Sigma type XIV) for 30 s to soften the ganglionic sheath, to aid penetration with intracellular electrodes and micropipettes. Intracellular recording was carried out from the left half of the terminal ganglion neuropil with glass microelectrodes filled with 2 M potassium acetate (electrode resistance from 30 to 50 MΩ). In some preparations, especially in the case of ascending interneurones, the electrode was filled with a 3% solution of Lucifer yellow CH in 0.1 M lithium chloride (electrode resistance ranged between 100 and 200 MΩ) to visualize neuron structure under a fluorescence microscope. Following physiological examination, the Lucifer dye was iontophoretically injected using 1–5 nA hyperpolarizing current pulses of 500 ms duration at 1 Hz for 2–10 minutes. Though each ascending interneurone was identified as a unique individual by its morphology (Nagayama et al., ’94), identification of individual nonspiking interneurones was difficult, since many interneurones showed similar gross morphologies (Nagayama et al., 2004). The uropod motor neurons and the nonspiking local interneurones were identified physiologically according to criteria that have been described previously (Nagayama et al., ’84, ’97).

GABA, L-Glutamate, and picrotoxin (PTX) were obtained from Sigma, and dissolved in normal crayfish saline to the required concentration. A 50% chloride saline was prepared by replacing NaCl in normal saline with equimolar amounts of sodium propionate (Albert et al., ’86). For bath application of 50 μM PTX or low chloride saline, the chamber (8 ml volume) was constantly perfused with fresh saline at a rate of 4 ml/min using a microtube pump (MP–3; Eyela, Tokyo, Japan). After physiological characterization, interneurons or motor neurons were rested for more than 3 min under a continuous perfusion of normal saline. Drugs were then perfused for 5 min, and washed out with normal saline. Small quantities of 1 mM GABA, or 0.5 or 1 mM glutamate were applied via pressure microinjection from micropipettes in the lateral neuropil of the terminal ganglion near the intracellular recording site. The tips of micropipettes were broken manually under a microscope to about 5 μm in outer diameter, and GABA or glutamate injected from the penetrated micropipette with nitrogen gas controlled by a pneumatic picopump (PV830, WPI) at 10–20 psi for 100–1,000 ms. Experimental arrangement of this paper was schematically drawn out in Figure 1.

Nonspiking interneurons are classified into two major groups, posterolateral (PL) and anterolateral (AL) interneurons, by their gross morphology and the position of their cell bodies (Nagayama et al., ’97). There were no significant differences in
results between PL and AL interneurons. All recordings were stored on a PCM data recorder and displayed on a Gould electrostatic chart recorder. The results are based on 65 successful intracellular recordings.

RESULTS

GABA- and glutamate-mediated hyperpolarization of nonspiking interneurons

When 1 mM GABA was applied locally into the lateral neuropil in the terminal ganglion near the branches of nonspiking interneurons, the spontaneous spike discharge of the opener motor neurons was completely suppressed (1st trace in Fig. 2A), and nonspiking interneurons showed a sustained membrane hyperpolarization (2nd trace in Fig. 2A). The amplitude of the hyperpolarization of the interneurons ranged from 5 to 25 mV depending on the preparation (n = 30), partly because of the different position of the micropipettes within the neuropil, and lasted for over 2 s in response to a 100 msec pulse of GABA injected into the neuropil. The input resistance of the nonspiking interneurons, measured by brief pulses of 1 nA hyperpolarizing current, was usually reduced by 10–30% (26.5 ± 4.7% mean ± SEM; n = 5) during the GABA-mediated hyperpolarization (Fig. 2B).

Local injection of L-glutamate into the neuropil also caused a sustained membrane hyperpolarization of the nonspiking interneurons. For example, the nonspiking interneuron shown in Figure 2C showed a sustained hyperpolarization of about 20 mV in amplitude when 0.5 mM glutamate was locally injected into the neuropil. The glutamate-mediated hyperpolarization of the nonspiking interneurons was variable in amplitude, from 5 to 20 mV (n = 6). The input resistance of the interneurons was also reduced by 30–60% (44.8 ± 9.5% mean ± SEM; n=3) during the glutamate-mediated hyperpolarization (Fig. 2D). There was no significant statistical difference in reduction of input resistance between GABA- and glutamate-mediated hyperpolarization.

The glutamate-mediated hyperpolarization of the nonspiking interneurons was reversed by the injection of 1 or 2 nA hyperpolarizing current (n = 6; Fig. 3C). For example, in the nonspiking interneuron shown in Figure 3A, a 1 nA hyperpolarizing current was sufficient to reverse the glutamate-mediated hyperpolarization. As the intensity of the injected current was increased, the potential progressively increased in amplitude. By contrast, the GABA-mediated hyperpolarization

Fig. 1. Schematic diagram of the experimental arrangement. See text in detail.
was reversed in less than half of the nonspiking interneurons (14 out of 30 interneurons) when 1 or 2 nA hyperpolarizing current was injected (Fig. 3C). In 9 interneurons, the amplitude of the GABA-mediated hyperpolarization was reduced, but was not reversed by the passage of a 3 nA hyperpolarizing current (Fig. 3B). Some interneurons were not reversed, even when 5 nA hyperpolarizing current was injected. The reversal potentials of the glutamate- and GABA-mediated inhibition of the nonspiking interneurons were clearly different ($p < 0.05$: Fisher's exact probability test).

**Effect of picrotoxin on GABA- and glutamate-mediated inhibition**

Picrotoxin (PTX) is a known non-competitive GABAA antagonist in vertebrates (Kaila, '94). Bath application of 50 μM PTX reduced the amplitude of the glutamate-mediated hyperpolarization of the nonspiking interneurons ($n = 3$), while it had no effect on the GABA-mediated hyperpolarization ($n = 13$).

The resting membrane potential of the nonspiking interneuron shown in Figure 4A was slightly depolarized after bath application of PTX. Local injection of glutamate reduced the hyperpolarization to less than half the initial size. After wash out with normal saline for about 20 min, the resting membrane potential of the interneuron shifted more negatively, while local injection of glutamate caused a reversed depolarizing response to the interneuron. Since stable and long-lasting (over 1 hour) intracellular recordings from nonspiking interneurons were technically difficult, owing to the small diameter of branches, resting potentials in many preparations did not recover fully before the condition of the electrode became unstable. In some experiments, therefore, the
effect of PTX on glutamate-mediated inhibition of ascending interneurons was examined. Identified ascending interneuron RC–5 (Nagayama et al., '94) showed membrane hyperpolarization of about 10 mV in amplitude when 1 mM glutamate was applied. The amplitude of membrane hyperpolarization of RC–5 was reduced after bath application of PTX that was recovered after 60 min of washing (Fig. 4B).

By contrast, the GABA-mediated hyperpolarization of the nonspiking interneuron did not reduce in amplitude after bath application of PTX (Fig. 5A). When PTX was applied, the size of the GABA-mediated hyperpolarization of many nonspiking interneurons was similarly not reduced in amplitude during bath application of a low chloride saline (8 out of 10 interneurons). After the saline was changed to a low chloride solution by replacement of Cl− with sodium propionate, the GABA-mediated hyperpolarization of the nonspiking interneuron shown in Figure 5B did not decrease significantly. A reversible reduction of the GABA-mediated hyperpolarization under low chloride solution was only observed in two nonspiking interneurons (Fig. 5C).

GABA-mediated inhibition of uropod motor neurons

Both GABA and glutamate also inhibited the spike discharge of the uropod opener motor neurons when they were injected locally into the neuropil (upper traces in Fig. 6A and B). Bath application of 50 μM PTX had no significant effect on the GABA-mediated inhibition of the opener motor neurons (middle trace in Fig. 6A). By contrast, the glutamate-mediated inhibition of the opener motor neurons began to decrease gradually after approximately 3 min bath
application of PTX. After 6 min (1 min after wash out) from PTX application, glutamate injection increased the spike frequency of the opener motor neurons (middle trace in Fig. 6B). Furthermore, the phasic spikes of the fast motor neuron with large amplitude in extracellular recordings were also observed just after glutamate was applied. The excitation of the opener motor neurons continued for several minutes, then the inhibitory effect of glutamate recovered gradually. Glutamate caused an inhibition of the opener motor neurons after about 30 min of washing (bottom trace in Fig. 6B).

The spike activity of the opener motor neurons was inhibited by electrical stimulation of sensory afferents innervating hairs on the surface of the exopodite. This inhibition of the motor neurons was not affected by PTX, since sensory stimulation also evoked a sustained inhibition of the spikes of the motor neurons under bath application of PTX (Fig. 6C). Intracellular recording from the fast opener motor neuron revealed that the ipsps of the motor neuron elicited by sensory stimulation were not decreased in amplitude under bath application of PTX (Fig. 7A). Local injection of 1 mM GABA into the neuropil also caused a hyperpolarization of the motor neuron. Bath application of PTX had similar no effects on sensory stimulation (Fig. 7B).

**DISCUSSION**

The results demonstrated that local injection of GABA and glutamate mediated a membrane hyperpolarization of the nonspiking and ascending interneurons in the terminal abdominal ganglion of crayfish. Every interneuron tested showed an inhibitory response to GABA or glutamate injection. The glutamate-mediated inhibition was PTX-sensitive, while the GABA-mediated inhibition was PTX-insensitive. Furthermore, bath application of low chloride solution usually had no effect upon the GABA-mediated hyperpolarization of the interneurons.
Glutamate as an inhibitory transmitter

Although glutamate is generally considered to be an excitatory neurotransmitter at neuromuscular junctions (Takeuchi and Takeuchi, '63), it is now well-established that it also mediates inhibitory synaptic transmission of crustacean motor neurons (Marder, '87). Like GABA application, pressure injection of a small amount of glutamate into the neuropil inhibits many stomatogastric motor neurons (Marder and Paupardin-Tritsch, '78; Tazaki and Chiba, '94), leg motor neurons (Pearlstein et al., '94, '98), abdominal postural motor neurons (Heitler et al., 2001), and swimmeret motor neurons (Sherff and Mulloney, '96). Glutamate also inhibits sensory afferents (Cattaert and LeRay, '98) and intersegmental ascending interneurons (Nagayama et al., 2004) of the crayfish. Furthermore, this study also demonstrated that glutamate mediated a

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**Fig. 5.** Effect of PTX and low-chloride saline on the GABA-mediated hyperpolarization of the nonspiking interneurons. A. The GABA-mediated hyperpolarization of a nonspiking interneuron was not reduced significantly following PTX perfusion for 5 min. After 40 min of washing, the GABA-mediated hyperpolarization was similar in amplitude to the control. B-C. After the saline was changed to a low-chloride solution, the GABA-mediated hyperpolarization of the interneurons was not decreased (B) or decreased (C) in amplitude. After more than 45 min of washing, the GABA-mediated membrane hyperpolarization of the interneuron in C recovered to initial control level. Recordings shown in A, B, and C are from different preparations.
hyperpolarization of nonspiking interneurons, as well as uropod motor neurons. Thus, glutamate is a common inhibitory transmitter that is frequently used in the central nervous systems of crustaceans.

Different ionic mechanisms underlying glutamate- and GABA-mediated inhibition

Both GABA and glutamate mediate inhibition of nonspiking interneurons, but their actions were
clearly different when 50 μM PTX was applied. PTX reduced the amplitude of the glutamate-mediated hyperpolarization of the nonspiking interneurons, while it had no effect on the GABA-mediated hyperpolarization. In many nonspiking interneurons, the GABA-mediated hyperpolarization was similarly not affected by the exchange of external solution to low chloride saline. In this study, the exact value of the reversal potential of both the glutamate- and GABA-mediated membrane could not be measured. Current injection experiments showed that the glutamate-mediated hyperpolarization was always reversed by 1–2 nA hyperpolarizing current injection, but more than 3 nA hyperpolarization was needed to reverse more than half of the GABA-mediated hyperpolarization (Fig. 3). Since the resting membrane potential of the nonspiking interneurons ranges between 40 and 55 mV (Nagayama et al., '84), the input resistance is about 10–20 MΩ (Hikosaka and Takahata, '98), and $E_K$ is approximately $-100$ mV, while $E_CL$ is $-65$ mV (Miwa et al., '86; Pearlstein et al., '94), the GABA-mediated inhibition of specific interneurons, as well as the glutamate-mediated inhibition, could be related to the gating of a Cl$^-$ conductance. Since the GABA-mediated hyperpolarization of many nonspiking interneurons was not reduced by low-chloride solution and their reversal potential was considerably negative from their resting membrane potential, their GABA-mediated inhibition appears to be K$^+$-dependent. Further studies using low potassium solution or K-channel blockers would be necessary to clarify this point.

Many investigators have reported the pharmacological profile of crustacean GABA and glutamate receptors. For example, crayfish leg motor neurons have nonspecific GABA/glutamate receptors linked to chloride channels that are PTX-sensitive (Pearlstein et al., '94, '98; Le Bon-Jego and Cattaert, 2002). Crayfish swimmeret motor neurons also have Cl$^-$-dependent GABA and glutamate receptors, though glutamate-mediated inhibition is PTX-sensitive but GABA-mediated inhibition is PTX-insensitive (Sherff and Mullooney, '96). Furthermore, crayfish leg sensory afferents have PTX-sensitive GABA receptors, but PTX-insensitive glutamate receptors (Cattaert and Le Ray, '98). In stomatogastric motor neurons, several types of GABA and glutamate receptors with different pharmacological profiles have also been characterized (Marder and Pau-pardin-Tritsch, '78; Tazaki and Chiba, '94). This variability of pharmacological profile of GABA and glutamate receptors in distinct classes of neurons might be due to their different functional roles within local circuits.

Under bath application of PTX, the tonic spikes of the opener motor neurons increased by local injection of glutamate (Fig. 6B). This suggests the possibility that glutamate also acts as an excitatory transmitter in the central nervous system, since glutamate elicited excitatory junctional potentials in crayfish adductor muscles (Nagayama et al., 2004) and excitatory responses in crab stomatogastric motor neurons (Marder and Pau-pardin-Tritsch, '78). The inhibitory effect of glutamate appears to be predominant, but an excitatory action might be important locally at certain central synapses.

**Functional differences in GABA- and glutamate-mediated inhibition**

Nonspiking interneurons received inhibitory inputs from spiking and/or nonspiking local interneurons (Nagayama, '97; Namba and Nagayama, 2004). Double labeling of intracellular staining and immunocytochemical labeling showed that many spiking local interneurons are glutamatergic (Nagayama et al., 2004), while nonspiking interneurons are mainly GABAergic (Nagayama et al., '97). Thus, nonspiking interneurons could receive both GABAergic and glutamatergic inhibitory inputs. Since PTX-sensitive ipsps are short-duration, while PTX-insensitive ipsps are long-duration (Tazaki and Chiba, '94; Miyata et al., '97), the excitability of the nonspiking interneurons would be more rigidly controlled by interactions between GABAergic and glutamatergic inhibition through parallel inhibitory pathways.

Since neither the sensory-evoked ipsps nor the GABA-mediated hyperpolarization of the uropod opener motor neurons were reduced in amplitude under bath application of PTX (Fig. 7), the sensory inhibition of the opener motor neurons is likely to be mediated through GABAergic synaptic transmission. High-frequency spike trains of spiking local interneurons are necessary to cause a sustained inhibition of motor neurons (Nagayama et al., '93). By contrast, small changes in the membrane potential of nonspiking interneurons are sufficient to continuously inhibit motor neurons (Nagayama et al., '94). Many nonspiking interneurons are GABAergic (Nagayama et al., '97), while no GABAergic spiking local interneurons have been identified (Aonuma and Nagayama, '99). Thus, nonspiking interneurons would be the
predominant premotor elements to form the motor pattern of the uropods.

**LITERATURE CITED**


