Distribution of Glutamatergic Immunoreactive Neurons in the Terminal Abdominal Ganglion of the Crayfish

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ABSTRACT

Using an antiserum directed against glutamate, we have analyzed the distribution of glutamate-like immunoreactive neurons in the terminal abdominal ganglion of the crayfish Procambarus clarkii. Approximately 160 central neurons (157 ± 8; mean ± SEM, n = 8) showed positive glutamate-like immunoreactivity, which represents approximately 25% of the total number of neurons in the terminal ganglion. Using a combination of intracellular staining with the marker Lucifer yellow and immunocytochemical staining has shown that most excitatory motor neurons are glutamatergic and that glutamate acts as an excitatory transmitter at peripheral neuromuscular junctions. Seven of 10 identified spiking local interneurons and only 2 of 19 identified ascending interneurons, showed positive immunoreactivity. Our observation that inhibitory spiking interneurons were immunopositive, whereas excitatory ascending interneurons were immunonegative, indicates that glutamate is likely to act as an inhibitory neurotransmitter within the central nervous system. Local pressure injection of L-glutamate into the neuropil of the ganglion caused a hyperpolarization of the membrane potentials of many interneurons. γ-Aminobutyric acid (GABA)ergic posterolateral nonspiking interneurons and the bilateral nonspiking interneuron LDS showed no glutamate-like immunoreactivity, whereas non-GABAergic anterolateral III nonspiking interneurons showed glutamate-like immunoreactivity. Thus, not only GABA but also glutamate are used in parallel as inhibitory neurotransmitters at central synapses. J. Comp. Neurol. 474:123–135, 2004. © 2004 Wiley-Liss, Inc.

Indexing terms: glutamate; double labeling; identified neurons; GABA; inhibitory transmitter

To understand in detail the neural circuitry underlying animal behavior, it is essential to identify the neurons involved and to reveal their patterns of interconnection. Furthermore, characterization of the neurotransmitters released from the presynaptic neurons and the receptor types on the postsynaptic neurons is essential to understand the functional properties of the circuits. Immunocytochemical staining, in particular combined with double labeling using intracellular markers, is a powerful method for characterizing the neurotransmitters of identified neurons (Reaves et al., 1982; Nagayama et al., 1997).

Arthropods have relatively simple nervous systems, and many of their neurons are identifiable as unique individuals. For example, the crayfish terminal abdominal ganglion contains approximately 650 central neurons, including 230 motor neurons, 130 ascending interneurons, and 290 local interneurons of both spiking and nonspiking types (Reichert et al., 1982; Kondoh and Hisada, 1986), and many of these neurons have been identified both physiologically and morphologically (Nagayama et al., 1993a,b; Wine, 1984). Many also comprise the local circuitry for uropod motor control and/or abdominal postural...
control, and the role of specific individual neurons has been analyzed in detail in avoidance and escape behaviors (Nagayama, 1997; Edwards et al., 1999). The neurotransmitters used by some of the neurons in the terminal abdominal ganglion have also been revealed. For example, acetylcholine is an excitatory transmitter that is released from sensory afferents (Ushizawa et al., 1996), whereas γ-aminobutyric acid (GABA) is an inhibitory transmitter released from many of the nonspiking local interneurons (Nagayama et al., 1997), some ascending intersegmental interneurons (Aonuma and Nagayama, 1999), and inhibitory motor neurons (Nagayama, 1999). Immunocytochemical studies have shown that approximately 10% of the total number of neurons in the terminal abdominal ganglion are cholinergic (Braun and Mulloney, 1994), and a further 10% are GABAergic (Mulloney and Hall, 1990; Nagayama et al., 1996). In addition, studies using antibodies against serotonin, octopamine, histamine, and protocollin have shown that fewer than 10 serotoninergic, octopaminergic, and histaminergic neurons, and approximately 20 protocolinergic neurons originate in the terminal ganglion (Siwicki and Bishop, 1986; Real and Czternasty, 1986). In our previous studies (Aonuma and Nagayama, 1999) showed that approximately 25% of the total number of neurons in the terminal abdominal ganglion are glutamatergic. Excitatory motor neurons predominantly show glutamate-like immunoreactivity, and known inhibitory interneurons that do not show GABA immunoreactivity show positive immunoreactivity against glutamate. Our results suggest that L-glutamate is a widely used transmitter in the terminal ganglion and acts as an excitatory transmitter peripherally, but also as an inhibitory transmitter within the central nervous system.

**MATERIALS AND METHODS**

Adult crayfish *Procambarus clarkii* Girard of both sexes (7–9 cm in length from rostrum to telson) were used in all experiments. They were obtained from a commercial supplier, maintained in laboratory tanks, and fed weekly on a diet of chopped potato and liver.

**Dissection and intracellular staining**

The abdomen was isolated from the thorax and pinned ventral side up in cooled van Harreveld’s (1936) solution. The terminal (sixth) abdominal ganglion was exposed by removing the sixth sternite and peeling off the ventral aorta and connective tissue. The soft cuticle overlying the uropod muscles was then removed along with the appropriate portions of the protopodite and the exopodite to allow access to nerve root 2. The spike activity of the closer, reductor motor neurons was recorded extracellularly by using a suction electrode on the second nerve root, just distal to the bifurcation to the reductor and adductor exopodite muscles (Nagayama et al., 1983). The terminal ganglion was stabilized on a silver platform and treated with protease (Sigma type XIV, Sigma, St. Louis, MO) for approximately 30 seconds. Intracellular recordings were made from the left half of the ganglion with glass microelectrodes filled with a 3% solution of Lucifer yellow CH in 0.1 M lithium chloride (electrode resistance ranged between 80 and 200 MΩ), and neurons were impaled in their neuropil processes or axons. The output effects of the impaled neurons on the activity of the reductor motor neuron were characterized by a change of their tonic spike number during 1–3-nA depolarizing current injection. Each neuron was then stained intracellularly by the iontophoretical injection of Lucifer yellow (1–7-nA hyperpolarizing current pulses of 500 msec duration at 1 Hz for 5–15 minutes). The ventral ganglionic sheet of the terminal ganglion was then surgically removed by using fine forceps, and the gross morphology of the stained neuron was confirmed in situ by using brief illumination of blue-violet light from a high-pressure mercury lamp. The stained neurons were identified both by their output effects on the reductor motor neuron and by their gross morphology, according to criteria described elsewhere (Nagayama, 1999; Nagayama et al., 1993a,b, 1994, 1997, 2002). Local application of glutamate into the neuropil of the terminal ganglion was performed by a pressure-
ejected micropipette controlled by a pneumatic picopump (Miyata et al., 1997).

**Immunocytochemistry**

The nerve chain, including the last two (fifth and sixth) abdominal ganglia, were isolated and fixed for 20 minutes in a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in Dulbecco’s phosphate-buffered saline (DPBS; Sigma), pH 7.4–7.5, at 4°C. The tissue was then washed for 60 minutes in several changes of wash buffer A (0.3% Triton X-100 [Sigma] in DPBS) at 25°C and stored at 4°C overnight. The primary antibody, mouse monoclonal anti-glutamate (Incstar, Stillwater, MN), at a dilution of 1:1,000, was preincubated with dried crayfish nerve and muscle powder (Mulloney and Hall, 1990) for 6 hours at 4°C in wash buffer B (0.3% Triton X-100 and 5% goat serum [Chemicon, Temecula, CA] in DPBS). The solution was centrifuged for 10 minutes, and the supernatant was collected. The nerve tissue was preincubated in several changes of wash buffer B for 60 minutes at 25°C and then incubated in the primary antibody solution for 6 hours at 25°C or overnight at 4°C on a rotator. The tissue was then washed in several changes of wash buffer A for 1 hour. Anti-mouse immunoglobulin G (IgG) indocarbocyanine (Cy3; Sigma) at a dilution of 1:1,500 in wash buffer A was used as the secondary antibody. The tissue was incubated in this secondary antibody solution for 6 hours at 25°C, or overnight at 4°C on a rotator and then washed in several changes of wash buffer A for 1 hour at 25°C. The tissue was then dehydrated in an ascending alcohol series, 50-70-80-90-95-100% for 10 minutes each and cleared in methyl salicylate.

Fluorescence was detected by using a fluorescence microscope (Olympus, Tokyo, Japan). The light employed for excitation was passed through 450–490-nm (for Lucifer yellow) and 510–560-nm (for Cy3) bandpass excitation filters, and the resulting fluorescence was passed through LP 520 (for Lucifer yellow) and LP 590 (for Cy3) barrier filters attached to the fluorescence microscope. Images were recorded in the same focal plane using a cooled CCD video camera (C4742-95, Hamamatsu Photometrics, Hamamatsu, Japan) and analyzed with the aid of Aqua-Cosmos image acquisition and analysis software system for videomicroscopy (Nikon, Tokyo, Japan). In some prep-
Fig. 3. A–C: Glutamate-like immunoreactivity of the motor giant (MoG) motor neuron. A: Gross morphology of MoG viewed from the ventral side. Anterior is to the top. B: Distribution of cell bodies with glutamate-like immunoreactivity. The cell body of MoG shown in A is indicated by an arrowhead. C: The image shown in B is superimposed on the reversed image of the Lucifer yellow-filled cell body of MoG, in the same focal plane. D–F: The uropod common inhibitory motor neuron (CI) was immunonegative. D: Gross morphology of the uropod CI viewed from the ventral side. Anterior is to the top. A bifurcated axon (indicated by arrows) runs in nerve root 1 and root 2. E: Distribution of cell bodies with glutamate-like immunoreactivity. F: The image shown in E is superimposed on the reversed image of the Lucifer yellow-filled cell body of the root 6 motor neuron, in the same focal plane. Anterior is to the top. G: Gross morphology of the root 4 motor neuron viewed from the ventral side. Anterior is to the top. The axon (indicated by arrow) runs in nerve root 4. H: Distribution of cell bodies with glutamate-like immunoreactivity. I: The image shown in H is superimposed on the reversed image of the Lucifer yellow-filled cell body of the root 4 motor neuron, in the same focal plane. The cell body of this motor neuron is not labeled in H. J–L: Non-glutamate-like immunoreactivity of the root 4 motor neuron. J: Gross morphology of a root 4 motor neuron viewed from the ventral side. Anterior is to the top. K: Distribution of cell bodies with glutamate-like immunoreactivity. L: The image shown in K is superimposed on the reversed image of the Lucifer yellow-filled cell body of the root 6 motor neuron, in the same focal plane. The cell body of the root 6 motor neuron is not labeled in K. The cell bodies of immunopositive neurons used as landmarks are indicated by asterisks to show the relative positions of the Lucifer yellow-filled cell body in E, F, H, I, K, and L. In E, the cell body of strong immunopositive neurons is indicated by +++, and that of weak immunoreactive neuron is indicated by +/-.

Scale bars = 100 μm.
arations, the Zeiss confocal microscope was also used to confirm immunoreactivity of the Lucifer yellow-filled neurons. Data are based on 163 double-labeling experiments of intracellular stains and immunocytochemical staining against glutamate from 37 motor neurons, 29 nonspiking local interneurons, 30 spiking local interneurons, and 67 intersegmental ascending interneurons. Interneurons that were encountered only once are not described in this study.

RESULTS

Immunocytochemical staining against anti-glutamate showed that approximately 25% of the total number of 600–700 cell bodies in the terminal abdominal ganglion of the crayfish were glutamatergic. Figure 1 gives a typical example of the distribution of cell bodies showing glutamate-like immunoreactivity in the terminal abdominal ganglion. Approximately 160 labeled cell bodies were counted on the ventral surface of the ganglion (157 ± 8; mean ± SEM, n = 8), whereas no labeled cell bodies were distributed on the dorsal surface.

Glutamate-like immunoreactivity of motor neurons

L-glutamate has been shown to be an excitatory transmitter at the neuromuscular junction of crustaceans and insects (Anwyl and Usherwood, 1974; Takeuchi and Takeuchi, 1963). When 0.1 mM L-glutamate was applied locally onto the adductor exopodite muscle by using pres-
Figure 5
ure ejection, a depolarizing excitatory junctional potential (EJJP) was evoked in the adductor muscle (Fig. 2A). This muscle was innervated by an identified adductor motor neuron whose cell body of about 50 μm in diameter was located in an anterolateral region of the terminal ganglion (Fig. 2B). Double labeling using a combination of intracellular staining (Fig. 2B) and subsequent immunocytochemical staining against glutamate (Fig. 2C) showed that the Lucifer yellow-stained cell body of the adductor motor neuron expressed glutamate-like immunoreactivity (arrowhead in Fig. 2C). To demonstrate this more clearly, the image of the cell body of the adductor motor neuron stained with Lucifer yellow was reversed and superimposed on that showing immunocytochemical staining. The resulting image clearly shows that the cell body of the adductor motor neuron was strongly immunopositive (cf. Fig. 2C and D).

To confirm the selectivity of the glutamate immunoreactivity, motor neurons whose physiology and neurochemistry are known were examined by double labeling. For example, the motor giant (MoG) motor neuron, which is known to be glutamatergic (Aonuma et al., 1999), has a large-diameter cell body (over 70 μm) in an anteromedial region of the ganglion near the midline, and a thick axon that projects peripherally through nerve root 6 (Fig. 3A). The Lucifer yellow-stained cell body of MoG showed strong glutamate-like immunoreactivity (arrowhead in Fig. 3B). On the other hand, the uropod common inhibitory neuron (CI) and the flexor inhibitory motor neuron (FI) are both known to be GABAergic inhibitory motor neurons (Nagayama, 1999). CI had a cell body of about 40 μm in diameter located in a posterolateral region of the ganglion, near the midline (Fig. 3D), and a bifurcating axon in nerve root 1 and root 2 (indicated by arrows in Fig. 3D). The Lucifer yellow-stained cell body of CI showed no glutamate-like immunoreactivity (cf. Fig. 3E and F). Similarly, the cell bodies of the FI showed no glutamate-like immunoreactivity (Table 1).

In total, 12 different motor neurons were examined by double labeling, and of the 10 non-GABAergic motor neurons stained with Lucifer yellow, 8 showed glutamate-like immunoreactivity, and 2 were immunonegative. One of these immunonegative motor neurons had an axon in the nerve root 4 motor bundle (indicated by arrow in Fig. 3G), a cell body of about 30 μm diameter located in a medial region near the midline (Fig. 3G), and expansive dendritic branches on both the soma and the contralateral side. Immunocytochemical staining against glutamate showed that several cell bodies around the cell body of the motor neuron were immunopositive (Fig. 3H), but the cell body of the motor neuron itself showed no glutamate-like immunoreactivity (Fig. 3I). The cell body of the second immunonegative motor neuron was located in a posterior region of the ganglion and had an axon in the nerve root 6 motor bundle (Fig. 3J). The primary neurite that exited from the cell body ran anteriorly and gave rise to posteriorly directed dendritic branches on the soma side and an unbranched process that crossed the midline to give rise to dendritic branches on the contralateral side. The Lucifer yellow-stained cell body of this root 6 motor neuron (reversed image in Fig. 3L) also showed no glutamate-like immunoreactivity (cf. Fig. 3K and L).

**Glutamate acts as inhibitory transmitter in the terminal abdominal ganglion**

Pearlstein et al. (1994) and Sherff and Mulloney (1996) have shown that L-glutamate acts as an inhibitory neurotransmitter within the central nervous system of crayfish. To confirm this possibility, L-glutamate was applied locally into the neuropil of the terminal abdominal ganglion, and the responses of interneurons were analyzed. Pressure injection of 1 mM L-glutamate completely suppressed the spontaneous spike discharge of the identified ascending interneuron, reciprocal opening ascending interneuron-5 (RO-5), and produced a sustained hyperpolarization of the membrane potential (Fig. 4A). At the same time, the spike discharge of the closer, reductor motor neuron was also completely inhibited. This glutamate-mediated hyperpolarization of RO-5 was reduced in amplitude by the injection of weak (1–2-nA) hyperpolarizing current and reversed by 3-nA hyperpolarizing current (Fig. 4A). Furthermore, the membrane conductance of the interneuron increased during the hyperpolarization, because the input resistance of the interneuron, measured by injection of brief pulses of 1-nA hyperpolarizing current, was reduced during the glutamate-mediated membrane hyperpolarization (n = 4; Fig. 4B). These results indicate that L-glutamate is likely to act as an inhibitory transmitter within the terminal ganglion.

**Glutamate-like immunoreactivity of nonspiking local interneurons**

Nonspiking local interneurons are the predominant premotor elements in the local circuitry of the terminal gan-
Figure 6
glion. They are divided into two major groups, PL (posterolateral) and AL (anterolateral) interneurons, according to their gross morphology and function (Nagayama and Hisada, 1987; Namba et al., 1994). AL interneurons are further subdivided into three types depending on the positions of their cell bodies. Previous studies have shown that all nonspiking interneurons, except for the AL-III type, are GABAergic (Nagayama et al., 1997).

Nonspiking interneurons of the AL-III group have an arched configuration with cell bodies located close to the anterolateral edge of the ganglion (Fig. 5A). Immunocytochemical staining showed that the Lucifer yellow-stained cell bodies of the AL-III interneurons (Fig. 5B) exhibited glutamate-like immunoreactivity (arrowhead in Fig. 5C).

Figure 5D–F shows magnified images of a single cell body (reversed image in Fig. 5F) plane through the cell body of the same interneuron under the confocal microscope. The Lucifer yellow-stained cell body (Fig. 5D and reversed image in Fig. 5F) was labeled by the antibody against glutamate (arrowhead in Fig. 5E).

In this study, we examined 11 AL-III interneurons, and 9 of these interneurons showed glutamate-like immunoreactivity (Table 1). Nonspiking local interneurons of the PL group have cell bodies located in a posterolateral region of the ganglion, and three subsets of PL interneurons have been identified by the number and pattern of projection of their main branches. A PL-1 interneuron was characterized by its main branch that ran posteriorly from the primary neurite, which then turned medially (Fig. 5G).

Immunocytochemical staining against glutamate (Fig. 5H) showed that the cell body of this interneuron (reversed image in Fig. 5I) showed no glutamate-like immunoreactivity (cf. Fig. 5H and I). The main branches of a PL-3 interneuron ran mainly anteriorly (Fig. 5J). Immunocytochemical staining against glutamate showed that two neighboring cell bodies with relatively large diameters were labeled (asterisks in Fig. 5K), but the cell body of this interneuron (reversed image in Fig. 5L) showed no glutamate-like immunoreactivity (cf. Fig. 5K and L).

Eight PL and two AL-II interneurons were examined in this study, but none showed positive glutamate-like immunoreactivity (Table 1). Furthermore, the bilateral sensory nonspiking interneuron (lateral directionally selective neuron [LDS]), which is GABAergic (Nagayama et al., 1996), showed no glutamate-like immunoreactivity (n = 8, Table 1).

**Glutamate-like immunoreactivity of spiking local interneurons**

Twenty spiking local interneurons have so far been identified both physiologically and morphologically, and they are divided into anterior, medial, and posterior groups based on the positions of their cell bodies (Nagayama et al., 1994). An interneuron of the anterior group, sp-ant1, had a cell body in an anterior midline location and main branches projecting posteriorly and laterally on the side contralateral to its cell body (Fig. 6A). One dorsal branch crossed the midline and turned anteriorly (arrow in Fig. 6A). This interneuron is known to make direct inhibitory connections with nonspiking interneurons (Nagayama, 1997) but shows no GABA-like immunoreactivity (Aonuma and Nagayama, 1999). The Lucifer yellow-stained cell body of sp-ant1 (Fig. 6B and reversed image in Fig. 6D) was labeled by immunocytochemical staining against glutamate (arrowhead in Fig. 6C). We have analyzed 10 identified spiking local interneurons using double labeling, and 7 of the 10 showed positive glutamate-like immunoreactivity. For example, the bilateral spiking local interneuron, sp-med5, had its cell body in an anteromedial region of the ganglion, near the midline, and an asymmetric pattern of branches on both sides, connected by an unbranched thin process (Fig. 6E). The Lucifer yellow-stained cell body of sp-med5 (Fig. 6F and reversed image in Fig. 6H) showed positive glutamate-like immunoreactivity (arrowhead in Fig. 6G). Another spiking local interneuron of the medial group, sp-med7, also had a cell body in an anteromedial region of the ganglion (Fig. 7A), but the Lucifer yellow-stained cell body (reversed image in Fig. 7C) showed no immunoreactivity (cf. Fig. 7B and C).

**Glutamate-like immunoreactivity of ascending interneurons**

Thirty ascending interneurons have so far been identified, both physiologically and morphologically (Nagayama et al., 1994). Nineteen of these interneurons were examined using double labeling, but 17 show no glutamate-like immunoreactivity (Table 1). For example, the ascending interneuron, reciprocal closing ascending interneuron-3 (RC-3), had a cell body in a rostrolateral region of the ganglion (Fig. 8A). This interneuron is known to make direct excitatory connections with nonspiking interneurons (Nagayama, 1997). The Lucifer yellow-stained cell body of RC-3 (reversed image in Fig. 8C) showed no immunocytochemical activity against glutamate (cf. Fig. 8B and C). Figure 8D shows an example of another ascending interneuron, variable effect ascending interneuron-1 (VE-1). The cell body of VE-1 is located in a very rostral region of the ganglion. This interneuron is known to make inhibitory connections with other ascending interneurons (Nagayama and Sato, 1993) and is labeled by immunocytochemical staining against GABA (Aonuma and Nagayama, 1999). Double-labeling staining showed that many glutamate-like immunoreactive cell bodies were labeled around the cell body of VE-1 (Fig. 8E), but the cell body of VE-1 was immunonegative (Fig. 8F). Two ascending interneurons, RC-4 and NE-1, showed glutamate-like immunoreactivity (Table 1). For example, NE-1 had a cell body located in the most caudal region of the ganglion and an axon of thick diameter in the anterior connective (Fig. 8G). The Lucifer yellow-stained cell body of about 50 μm...
in diameter (reversed image in Fig. 8I) showed positive glutamate-like immunoreactivity (arrowhead in Fig. 8H).

**DISCUSSION**

Our results are the first demonstration that many central neurons in the crayfish terminal abdominal ganglion show glutamate-like immunoreactivity. Histological studies have shown that the total number of cell bodies of central neurons in the terminal ganglion is approximately 650 (Reichert et al., 1982; Kondoh and Hisada, 1986). We show here that approximately 160 cell bodies stain positively against glutamate, which is about 25% of the total number of central neurons on the ventral surface of the terminal ganglion.

**Distribution of glutamate-like immunoreactive neurons in the terminal ganglion**

By using double labeling, we have analyzed the glutamatergic nature of 12 different motor neurons, 10 spiking local interneurons, 19 ascending interneurons, and 4 types of nonspiking interneurons.

There are 75 pairs of motor neurons whose axons exit the ganglion in nerve roots 1–6, and that innervate the uropod and/or telson muscles, and another approximately 70 motor neurons whose axons exit the ganglion in nerve root 7 that innervate the hindgut (Kondoh and Hisada, 1986). In this study, eight excitatory motor neurons (two root 1 dorsal rotator motor neurons, two root 2 uropod closer motor neurons, two root 3 uropod opener motor neurons, and two root 6 telson flexor motor neurons) showed glutamate-like immunoreactivity, whereas two inhibitory motor neurons and two unidentified motor neurons (root 4 and root 6 motor neurons) were not labeled. Because five GABAergic inhibitory motor neurons (one root 1-2 uropod common inhibitor, two root 3 motor neurons, and two root 6 flexor inhibitor motor neurons) have already been characterized in the terminal ganglion (Muloney and Hall, 1990; Nagayama, 1999), these two unlabeled motor neurons are not likely to be GABAergic. The motor neuron whose axon runs in nerve root 4 is known to be one of a bilateral pair stained in backfilling studies (Calabrese, 1976; Kondoh and Hisada, 1986, 1987), although its detailed morphology has until now never been described. Even though there are many studies describing telson postural motor neurons with axons running in nerve root 6 (Dumont and Wine, 1987), the muscle innervation of the unlabeled root 6 motor neuron found in our study remains unclear. At the moment, we do not know the function of these two nonglutamatergic motor neurons, but it is possible that another neurotransmitter besides glutamate and GABA could be used by these motor neurons.
Fig. 8. A–C: Non-glutamate-like immunoreactivity of excitatory ascending interneuron, RC-3. A: Gross morphology of RC-3 viewed from the ventral side. Anterior is to the top. B: Distribution of cell bodies with glutamate-like immunoreactivity. C: The image shown in B is superimposed on the reversed image of the Lucifer yellow-filled cell body of RC-3, in the same focal plane. The cell body of RC-3 is not labeled in B. D–F: Non-glutamate-like immunoreactivity of GABAergic ascending interneuron, VE-1. D: Gross morphology of VE-1 viewed from the ventral side. Anterior is to the top. E: Distribution of cell bodies with glutamate-like immunoreactivity. F: The image shown in E is superimposed on the reversed image of the Lucifer yellow-filled cell body of VE-1 in the same focal plane. The cell body of VE-1 is not labeled in E. G–I: Glutamate-like immunoreactivity of ascending interneuron, NE-1. G: Gross morphology of NE-1 viewed from the ventral side. Anterior is to the top. H: Distribution of cell bodies with glutamate-like immunoreactivity. The cell body of NE-1 shown in G is indicated by an arrowhead. I: The image shown in H is superimposed on the reversed image of the Lucifer yellow-filled cell body of NE-1, in the same focal plane. The cell bodies of immunopositive neurons used as landmarks are indicated by asterisks to show relative position of the Lucifer yellow-filled cell body in B, C, E, and F. Scale bars = 100 μm.
Approximately 65 pairs of ascending interneurons originate in the terminal ganglion and send axons anteriorly. We have characterized the glutamate immunoreactivity of 19 identified ascending interneurons, and only 2, interneurons RC-4 and NE-1, show positive immunoreactivity. Indeed, few axons were observed in the A5–A6 abdominal connective even though a number of axons were strongly labeled in the motor nerve by immunocytochemical staining. Only a small number of ascending interneurons are therefore likely to be glutamatergic.

Six ascending interneurons (CI-2, RC-5, RO-6, VE-1, NE-2, and NE-4) have been shown to be GABAergic (Aonuma and Nagayama, 1999) and none of them showed strong glutamate-like immunoreactivity. About 20% of them (5 of 28 neurons), however, showed very weak staining that would be due to the cross-reactivity of Incstar’s primary antibody, because glutamate is a precursor of GABA. A similar relationship exists for the nonspiking local interneurons. GABAergic PL interneurons and LDS (Nagayama et al., 1996, 1997) show no strong glutamate-like immunoreactivity, whereas non-GABAergic AL-III interneurons show positive immunoreactivity against glutamate. Thus, there appears to be no strong cross-reaction with GABA during immunocytochemical staining against glutamate.

**Glutamate acts as an inhibitory transmitter in central synapses**

We studied 10 identified spiking local interneurons using double labeling, and 7 of them showed glutamate-like immunoreactivity. Because many spiking local interneurons are known to make direct inhibitory connections with nonspiking interneurons (Nagayama, 1997), it is therefore likely that glutamate is released by these neurons as an inhibitory neurotransmitter in the central nervous system. Although glutamate has been reported to activate glutamate extrajunctional receptors that increase chloride conductance in locust leg muscle (Dudel et al., 1989), this is in direct contrast to the effects of glutamate on peripheral neuromuscular junctions, where the application of glutamate caused an excitatory junctional potential in muscle fibers (Aonuma et al., 1999). Marder and Paupardin-Tritsch (1978) have reported that glutamate has both inhibitory and excitatory effects on central neurons in the crab stomatogastric ganglion, although the responses are mostly inhibitory. After their report, several crustacean systems in which the role of glutamate as an inhibitory transmitter were investigated intensively, including the stomatogastric nervous system (Tazaki and Chiba, 1994; Cleland and Selverston, 1995, 1997, 1998; Johnson and Harris-Warrick, 1997), the motor network controlling walking in crayfish (Pearlstein et al., 1994, 1998), and escape reflex in crayfish (Heitler et al., 2001). Because the localized injection of glutamate into the neuropil caused a hyperpolarization of many interneurons including the nonspiking interneurons, glutamate is likely to be a widely used inhibitory transmitter in the terminal ganglion. The observations that ascending interneurons, which make direct excitatory connections with nonspiking and/or ascending interneurons, do not show glutamate-like immunoreactivity support this idea.

**The role of glutamatergic neurons in the local circuitry**

This study demonstrates that the main subgroup of AL interneurons (AL-III) are glutamatergic, whereas the antagonistic PL interneurons are GABAergic (Nagayama et al., 1997). Furthermore, we also show that inhibitory spiking local interneurons are glutamatergic whereas inhibitory ascending interneurons are likely to be mainly GABAergic (Aonuma et al., 1999). Heitler et al. (2001) have recently showed that both GABA and glutamate mediate the inhibition of synaptic transmission at the electrical synapses between the giant fibers and motor giant (MoG) motor neuron in crayfish. What is the functional difference in the role of glutamate and GABA as inhibitory neurotransmitters in the local circuits within the terminal ganglion? At present, the answer remains unclear and suggests that further studies are necessary to characterize the inhibitory responses of the postsynaptic neurons to both GABA and glutamate, and to clarify the ionic properties of both GABA and glutamate receptors, in order to gain further insights into the neurochemical organization of local circuits.

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