

## Cellular distribution of a high-affinity glutamate transporter in the nervous system of the cabbage looper *Trichoplusia ni*

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### Summary

Glutamate functions as a neurotransmitter in the central nervous system (CNS) and neuromuscular junctions in insects. High-affinity glutamate transporters are responsible for keeping the resting levels of excitatory amino acids below the synaptic activation threshold by removing them from the extracellular fluid, thereby preventing them from reaching toxic levels. Peptides representing the N- and C-terminal regions of a glutamate transporter cloned from the cabbage looper caterpillar (*Trichoplusia ni*) were synthesized and used to generate polyclonal antibodies. The antibodies produced immunohistochemical staining in both muscular and nervous system *T. ni* tissues. Neuromuscular junctions in

the skeletal muscles produced the most intense labelling, but no visceral muscle or sensory nerves were labelled. In the CNS, the neuropile of the ganglia, but not the connectives, gave a diffuse staining. Electron microscopical examination of ganglia and neuromuscular junctions showed that the plasma membrane of glial cells, but not that of neurons was labelled, in agreement with the notion that most of the glutamate uptake sites in this insect are in glial cells.

Key words: glutamate transporter, *Trichoplusia ni*, insect, anti-peptide antibody, immunohistochemistry, glutamate.

### Introduction

Glutamate functions as a neurotransmitter in animals ranging from anemone, leech, earthworm, snail, squid and arthropods to mammals (Duce, 1988). In mammals, it is the predominant excitatory neurotransmitter in the central nervous system (CNS) (for a review, see Fonnum, 1984; Ottersen and Storm-Mathisen, 1984; Danbolt, 2001). Glutamate is neurotoxic in high concentrations (Choi, 1988) and has to be removed from the extracellular fluid. This is accomplished by cellular uptake catalysed by a family of sodium- and potassium-coupled glutamate transporters found in the plasma membranes of both neurons and glial cells (for reviews, see Danbolt et al., 1998a; Danbolt, 2001; Seal and Amara, 1999; Sims and Robinson, 1999). Five glutamate transporters, named excitatory amino acid transporters (EAAT) 1 to 5, have been identified in various mammalian tissues.

Glutamate uptake has also been demonstrated in neural and neuromuscular tissues in arthropods (Duce, 1988). In insects, glutamate has been shown to be a transmitter in the CNS and at neuromuscular junctions (Faeder and Salpeter, 1970; Usherwood and Machili, 1968; Beranek and Miller, 1968; Jan and Jan, 1976). Glutamate transporter cDNAs have been cloned from four orders of insects, namely Lepidoptera (the cabbage looper *Trichoplusia ni*; Donly et al., 1997),

Hymenoptera (the honeybee *Apis mellifera*; Kucharski et al., 2000), Diptera (the fruit fly *Drosophila melanogaster*; Seal et al., 1998; Besson et al., 1999) and Dictyoptera (the cockroach *Diploptera punctata*; Donly et al., 2000). The cabbage looper, cockroach and honeybee cDNAs were cloned from brain, and the fly cDNA from embryonic tissue. All these proteins show a high degree of amino acid identity and have comparable kinetic and pharmacological properties (Caveney and Donly, 2002). Compared with mammalian EAATs, the peptide sequence of the honeybee EAAT is most similar to mammalian EAAT2, while fruit fly EAAT1 is closest to mammalian EAAT1 and cockroach EAAT closest to mammalian EAAT3. A second *Drosophila* transporter has also been described (Besson et al., 1999), suggesting that the insect CNS may have multiple forms as seen in mammals. The *T. ni* transporter, designated TrnEAAT1 (*T. ni* excitatory amino acid transporter 1), shows considerable sequence identity to human EAAT-1 (40%), -2 (37%), -3 (42%) and -4 (39%), although it has the highest identity (up to 53%) to other cloned insect EAATs (Donly et al., 2000). Tissue expression of TrnEAAT1 has been analyzed by northern blot analysis and the highest levels were detected in extracts of RNA from brain, lower levels of expression in the integument,

and a weak expression in hindgut and rectum (Donly et al., 1997).

Since the cDNAs for most of these proteins were initially isolated using insect head or brain libraries, these transporters are most likely expressed in the CNS of these insects. Northern blot analysis has confirmed that the mRNAs for these proteins are synthesized in the CNS, optic lobes and possibly digestive tract of insects. However, these experiments do not give the precise localization of the protein, which may differ from the site of synthesis of its mRNA. Also, owing to the difficulty of isolating individual cell types in the preparation of cDNA libraries, the cells expressing the transporters have not been identified. Here we report on the cellular localization of the protein product of the TrnEAAT1 gene in tissues of the cabbage looper. Polyclonal antibodies were raised against peptide sequences in the C- and N-terminal regions of the TrnEAAT1 protein and used to localize the transporter. The transporter was found to be localized primarily at the neuromuscular junctions in skeletal muscle and in the ganglionic neuropile of the CNS.

## Materials and methods

### Animals

*Trichoplusia ni* (Huebner) caterpillars were raised on an artificial diet using standard procedures (16 h:8 h L:D photoperiod at 27 °C; Guy et al., 1985) at the Southern Crop Protection and Food Research Centre, London, Ontario, Canada.

### Antibodies

Anti-peptide antibodies against TrnEAAT1 were prepared as described (Lehre et al., 1995; Danbolt et al., 1998b) using synthetic peptides corresponding to residues 2–15 (PLQIRR-NRCTSFLR) and 450–479 (LSQGDIDKSRALNEREAAPS-HELTELEKGDH) of the sequence (Donly et al., 1997) as antigens. The former peptide was synthesized as a C-terminal amide, while the latter was made with free carboxyl terminus. Both peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde and injected into New Zealand White (female) rabbits. Rabbits 67JK, JP75, JQ19 and JQ31 were immunized with the N-terminal peptide, while rabbits 8D0145 and 8D0268 were immunized with the C-terminal peptide. The sera were affinity-purified on columns with immobilized peptide as described (Danbolt, 1998b). This study is based on sera from rabbits JQ31 and 8D0145, which yielded the best antibodies.

### Protein isolation

#### Virus-infected cells

Total protein extracts were prepared from High Five cells (BTI-TN-5B1-4, Invitrogen) infected with recombinant baculovirus expressing the TrnEAAT1 gene (Donly et al., 1997) and from uninfected control cells. Cells were harvested from the growth medium, centrifuged and resuspended in 10 mmol l<sup>-1</sup> sodium phosphate buffer and immediately frozen. Thawed cells were homogenized in cold phosphate buffer and

centrifuged at 16,000g for 40 min at 4 °C. The supernatant (water-soluble fraction) was collected and frozen and the pellet solubilized at room temperature in buffer containing 1 % SDS. The sample was centrifuged, the supernatant ('membrane' fraction) collected and frozen, and the pellet discarded.

### Insect tissues

*T. ni* caterpillars were anaesthetized in 75 % ethanol, and quickly dissected to isolate tissue samples. Caterpillar integument and associated skeletal muscle was briefly rinsed in buffer and then immediately frozen in liquid nitrogen. Adults were anaesthetized at 4 °C prior to isolation of flight muscle and heads, which were immediately frozen in liquid nitrogen. Thawed tissue samples were quickly homogenized in 10 volumes of phosphate buffer using a Brinkmann Polytron Homogenizer. Samples were then processed as described for High Five cells.

### Western blot analysis

Frozen samples containing 30 µg of protein per well were thawed, diluted in SDS-gel electrophoresis sample buffer [60 mmol l<sup>-1</sup> Tris base, pH 6.8, 25 % glycerol, 2 % sodium dodecyl sulfate (Sigma), 14.4 mmol l<sup>-1</sup> 2-mercaptoethanol (Sigma), 0.1 % Bromophenol Blue (Sigma)] and incubated at room temperature for 15 min. SDS-PAGE was performed as described by Laemmli (1970) on separating gels consisting of 8 % acrylamide. Protein molecular mass standards were Bio-Rad prestained SDS-PAGE broad range marker proteins. Gels were then electroblotted to nitrocellulose (Bio-Rad) membranes (Towbin et al., 1979). Membranes were blocked with 5 % nonfat milk in TBS (10 mmol l<sup>-1</sup> Tris base, 150 mmol l<sup>-1</sup> NaCl, pH 7.5) with 0.05 % Triton X-100 (Sigma) for 1 h at room temperature, transferred to primary antibodies (diluted to 1 µg/ml anti-C antibody and 30 µg/ml anti-N antibody) in the blocking buffer, and incubated for 24 h. The membrane was then washed in TBS (10 mmol l<sup>-1</sup>) for 1 h with changes, then incubated in anti-rabbit IgG (whole molecule)-alkaline phosphatase conjugate (1:30,000; Sigma) in blocking buffer for 4–24 h at 4 °C on a shaker. The membrane was washed three times in TBS, and then incubated for 15 min in alkaline phosphatase buffer (0.1 mol l<sup>-1</sup> Tris base, pH 9.5, 0.1 mol l<sup>-1</sup> NaCl, 5 mmol l<sup>-1</sup> MgCl<sub>2</sub>). Blots were developed in BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) liquid substrate (Sigma) to visualize the labelled bands. The reaction was stopped in 20 mmol l<sup>-1</sup> EDTA in TBS and the blots photographed.

### Isolation of tissues for light and electron microscopy

Third or fourth instar caterpillars were anesthetized for 30 s in 75 % ethanol, then dissected in cold 4 % paraformaldehyde/0.5 % glutaraldehyde in Tris (25 mmol l<sup>-1</sup>) buffered saline (TBS), pH 7.4. Adults were immobilized in the cold (4 °C) for 30 min then dissected in fixative as per caterpillars. Tissue samples were removed after 5 min and placed in fresh fixative at 4 °C for 4 h. The fixative was removed and the samples washed in TBS for 1 h at 4 °C.

### Light microscopy of nerve tissue

Caterpillars were chilled at 4°C for at least 1 h and then injected with 1% Methylene Blue in normal saline (Plotnikova and Nevmyvaka, 1980). The insects were left at room temperature for 4 h and then dissected in a fixative of 12% ammonium molybdate (aqueous). Tissue samples were excised and immediately examined in the light microscope.

### Immunohistochemistry

Tissues were incubated in TBS/0.1% Triton X-100 (TTBS) containing 1% bovine serum albumin (BSA) for 4 h at 4°C, then treated with primary antibody in TTBS/BSA for 8–24 h at 4°C on a shaker. After primary incubation, the samples were washed in TTBS for 4 h and then incubated with anti-rabbit IgG alkaline phosphatase conjugate (Sigma; diluted 1:400 in TTBS/BSA) for 4 h to overnight at 4°C. The tissues were washed in TTBS for 4 h at room temperature. Visualization of the probe was achieved with the indirect chromogenic method using one of the following substrates: Vector Red, Vector Blue, Vector Black (Vector Laboratories) and BCIP/NBT (Sigma). Although blocking for endogenous alkaline phosphatase in the insect tissues was not needed, the resultant reaction was checked by comparing it to the results obtained with secondary antibodies linked to rhodamine (Cappel Laboratories) or gold (British BioCell). Both of these non-enzymatic systems produced the same results as the alkaline phosphatase, confirming its reliability in this study. After the substrate produced a suitable colour reaction, the tissue was washed in water, mounted in Geltol Mounting Media (Immunon) and photographed with an Axiovert 35 photomicroscope (Zeiss) on Elite Chrome 160 T slide film (Eastman Kodak).

Silver enhancement of gold for light microscopy was performed by the method of Danscher (1981).

### Gold labelling

Fixed muscle tissues were washed in TBS at 4°C for 1–2 h, followed by a treatment in TTBS/1% BSA for 4 h. Specimens were incubated overnight in primary antibody (C-peptide antibody) diluted in TTBS/BSA, then washed in TBS for 5 h with solution changes, followed by incubation in goat anti-rabbit IgG 1 nm or 10 nm gold (British BioCell) diluted 1:100 (manufacturer's recommendations) in buffer for 24 h. After washing overnight in buffer, samples were re-fixed in 2% glutaraldehyde for 10 min and washed again in buffer. Tissues were then treated with 0.5% osmium tetroxide/TBS for 30 min (room temperature), washed with double-distilled water for 20 min and stained with 2% aqueous uranyl acetate for 30 min. Samples were washed in double-distilled water and dehydrated through a graded acetone series and embedded in Epon/Araldite. Sections were cut with a diamond knife on a Reichert ultramicrotome and mounted on nickel grids. Grids were examined in a Philips CM10 electron microscope.

Ganglia were fixed, dehydrated and embedded in epon as described above, except that the osmium tetroxide was omitted. Sections mounted on nickel grids were floated on a drop of saturated sodium metaperiodate for 10 min (room

temperature), and then washed three times in distilled water, followed by a treatment in 0.1 mol l<sup>-1</sup> HCl for 5 min. The grids were washed in PTBN buffer (8.5 g NaCl, 40 µl 0.5 mol l<sup>-1</sup> NaPO<sub>4</sub>, pH 7.4, 500 µl Tween 20, 1 g BSA l<sup>-1</sup> ddH<sub>2</sub>O) for 4 × 10 min, incubated in primary antibody (1/100–1/500) diluted in PTBN (overnight at room temperature), washed in buffer (4 × 1 min), and then treated with anti-rabbit immunoglobulin coupled to 15 nm gold (British BioCell) in PBTN (1/75) for 30 min. Finally, grids were washed in double distilled water and stained with 3% aqueous uranyl acetate for 30 min prior to examination in the electron microscope.

## Results

### Antibody specificity

Antibodies were raised in rabbits against residues 2–15 (N-terminal region) and residues 450–479 (C-terminal region) of the lepidopteran glutamate transporter TrnEAAT1 (Donly et al., 1997). Following affinity purification, the antibodies were tested on immunoblots of baculovirus-infected High Five cells to check if they recognized the TrnEAAT1 protein. Virus-infected and non-infected High Five cells were homogenized and separated into water soluble and membrane (water insoluble) fractions. These fractions were run on SDS-PAGE, and immunoblotted with the two antibodies (Fig. 1). Only the membrane fractions of the cells infected with the TrnEAAT1-expressing virus displayed any detectable immunoreactivity with the two antibodies.

The labelling patterns obtained with the two antibodies were the same, but the C-terminal antibody consistently exhibited

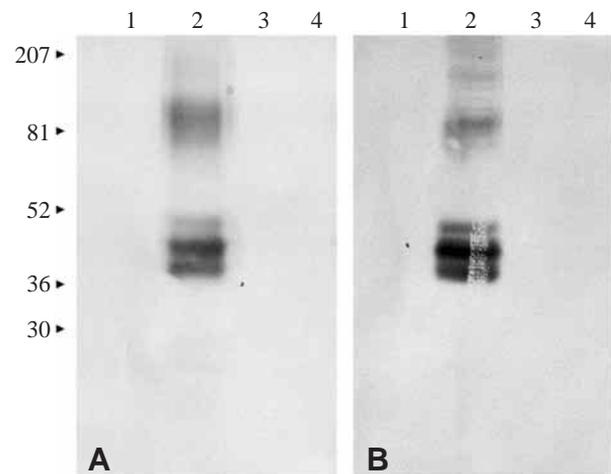


Fig. 1. Immunoblots (western blots) of crude protein extracts of High Five (H5) cells expressing TrnEAAT1 and non-expressing control cells probed with antibodies to the C-terminal (A) and N-terminal (B) peptide. The two antibodies produced similar patterns in the membrane fractions and failed to detect bands in the lanes containing the water-soluble fractions. Lanes: 1, H5-infected soluble fraction; 2, H5-infected membrane fraction; 3, H5 control water-soluble fraction; 4, H5 control membrane fraction. The positions of marker proteins (kDa) are shown.

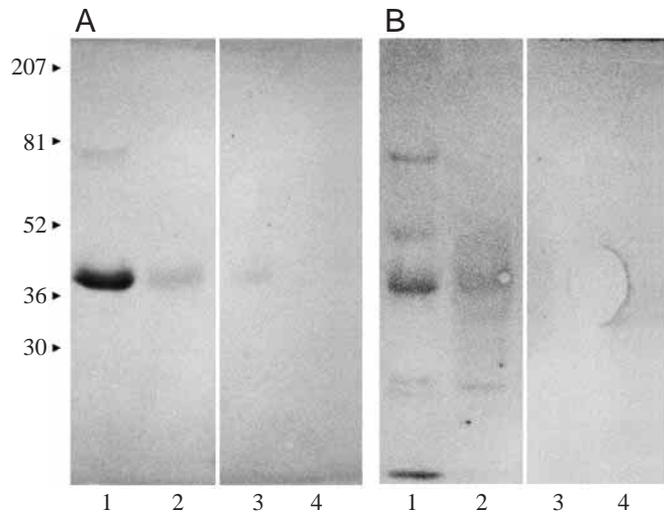


Fig. 2. Immunoblots (western blots) of caterpillar and moth tissue samples probed with the N- and C-terminal-specific TrnEAAT1 antibodies. (A) Tissue extracts probed with the antibody to the C-terminal peptide; (B) extracts probed with antibody to the N-terminal peptide. Lanes: 1, moth head membrane fraction; 2, caterpillar skeletal muscle membrane fraction; 3, moth head water-soluble fraction; 4, caterpillar skeletal muscle water-soluble fraction. The transporter bands are detected in the membrane fractions as in Fig. 1, and the positions of marker proteins (kDa) are shown.

the highest affinity for the TrnEAAT1 protein and as a result the N-terminal antibody had to be used at a higher concentration. The three main bands that were consistently visualized included two at 40–50 kDa and one at 80–100 kDa. The predicted molecular mass of the TrnEAAT1 protein is 52 kDa, so it is probable that the strongest band (nearer 50 kDa) represents the TrnEAAT1 monomer. The reason for this anomalous mobility on SDS-PAGE is not clear, although the redox state of the markers can affect apparent mobility relative to other transporters (Haugeto et al., 1996). This band also matches that found in insect tissues (Fig. 2). Because these kinds of protein easily form SDS-resistant complexes (Haugeto et al., 1996; Danbolt, 2001), the largest band at 80–100 kDa is most likely a TrnEAAT1 dimer (Fig. 1). The third and fastest migrating band may represent unglycosylated TrnEAAT1 since its mobility is greater than the product observed in insect tissue (which would be expected to be fully processed). A fourth faint product migrating between the TrnEAAT1 monomer and dimer was not consistently observed. Despite the heterogeneity of the products, these blots demonstrate specificity for the TrnEAAT1 protein at two levels. First, the only reactivity occurs in cells infected with the TrnEAAT1-expressing virus. Second, the same bands are recognized by two separate antibodies raised in different rabbits against two different peptides, one from each terminus of the *T. ni* glutamate transporter.

Having concluded that the antibodies recognize the TrnEAAT1 protein, the specificity of the antibodies was tested on immunoblots of crude preparations of *T. ni* tissues. Both

caterpillar skeletal muscle and adult head tissue probed with either antibody recognized a band between the 36 and 52 kDa markers (Fig. 2A,B). A high molecular mass oligomeric band was also present in the head tissue lanes, as seen in the immunoblots of extracts of virally infected High Five cells. Also consistent with the High Five blots, the major bands are detected only in the lanes with membrane proteins (although one very weak band can be seen in the adult head lane of a water soluble fraction; Fig. 2A, lane 3). Fig. 2A,B shows that the two antibodies specifically stain the TrnEAAT1 protein in both the head and body of *T. ni*. However, the anti-N serum, which had to be used at a higher concentration than the anti-C serum in order to visualize the bands, gave a less intense signal and higher background. Because of this all tissue staining experiments were performed with the anti-C serum, as it exhibited no crossreactivity in the tissue extracts.

#### Tissue distribution of TrnEAAT1

A whole-mount view of a caterpillar abdominal segment stained with the TrnEAAT1 C-terminal antibody is shown in Fig. 3. The branching pattern seen on the surface of the segmental skeletal muscle fibres was identified as a network of axoglial projections formed by motor neurons and associated glial cells (details below). The expression pattern of TrnEAAT1 shown in Fig. 3 shows that these stained projections are segmentally compartmented. The projections do not cross the segment border but instead terminate where the muscle inserts into the body wall (Fig. 3). This segmentally recurring and restricted pattern of antibody staining of skeletal muscle is found along the caterpillar's body. The ventral nerve

Table 1. Distribution of glutamate transporter TrnEAAT1 in *Trichoplusia ni* tissues

Tissues	Antibody binding
Caterpillar	
Nerve cord	+
Skeletal muscle	+
Foregut	–
Oesophagus	+
Midgut	–
Hindgut	–
Fat body	–
Trachea	–
Silk glands	–
Integument	–
Gonads	–
Heart	–
Adult	
Flight muscle	+
Skeletal muscle	+
Fat body	–
Integument	–

+, positive immunohistochemical staining reaction with antibody; –, no reaction.

cord in whole-mount preparations also stained with the TrnEAAT1 C-terminal antibody, but only the ganglia were seen to stain above background levels. The connectives and the nerve branches radiating out from the ganglia did not stain (Fig. 3). Residual tissues left after the dissection, such as fat body, trachea, and integument also did not stain.

Table 1 lists the distribution of the transporter protein in caterpillar tissues detected by the C-terminal antibody. Strong positive reactions were seen in skeletal muscle, flight muscle and the neuropile of ganglia. Some fibres in intra-oesophageal muscle stained in a pattern similar to that of the skeletal muscle. Midgut and hindgut tested negative. Isolated silk glands, gonads, fat bodies, trachea and integument did not react detectably to the antibody, and the muscles of the dorsal vessel and adjoining diaphragm also failed to react. Northern blot analysis had previously suggested that mRNA for TrnEAAT1 was expressed weakly in the integument, hindgut and rectum (Donly et al., 1997). The previous detection of TrnEAAT1 mRNA in the isolated integument (Donly et al., 1997) most likely resulted from contamination by muscle tissue.

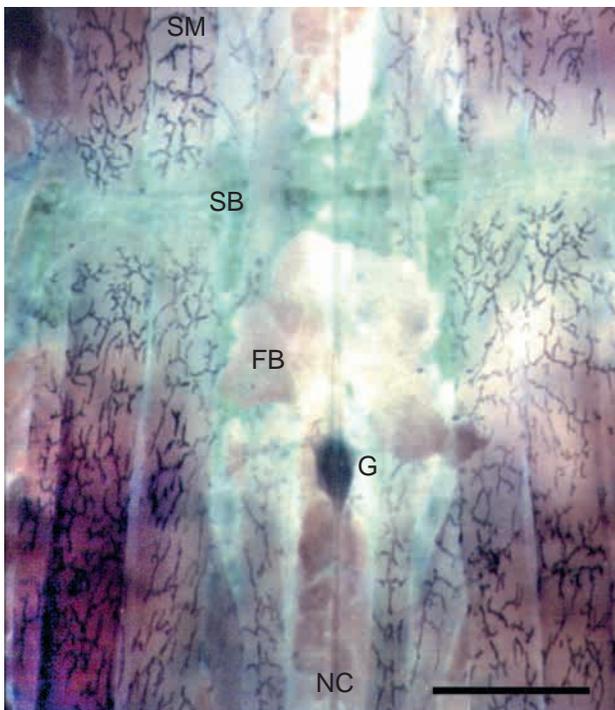


Fig. 3. Whole-mount view of an abdominal segment of a caterpillar stained with the TrnEAAT1 C-terminal antibody. A branched network of nerves found on the surface of the skeletal muscle fibres labelled strongly with this antibody. The stained projections of these nerves do not appear to cross the segmental borders. The only other structures labelled by the antibody were the ganglia. FB, fat body; G, ganglion; NC, nerve cord; SB, segmental boundary; SM, skeletal muscle. The tissue was probed with antibody directed to the C-terminal region of the transporter protein. A matching pattern was produced by the N-terminal antibody in separate preparations (not shown). Bound antibodies were visualized with the alkaline phosphatase substrates BCIP/NBT. Bar, 1 mm.

No reaction was seen in tissues treated with either pre-immune serum or when the primary antibody was omitted. The staining reaction was prevented when the antibody was treated with the peptide against which it was raised before use on insect tissues. A commercial anti-glutamate transporter antibody (Chemicon International), raised against the unique amino acid sequence of the C terminus of cloned rat EAAC1 (504–523) also failed to stain insect tissues. Finally, despite its lower affinity for TrnEAAT1 protein, treatment with the N-terminal antibody produced the same tissue staining patterns as those produced with the C-terminal antibody.

#### Muscle staining

TrnEAAT1 C-terminal antibody staining revealed a branched network of nerves (motor neurons and associated glial cells) that completely covered the surface of caterpillar skeletal muscle (Fig. 4). This is of special interest since previous work has shown that glial (sheath) cells at neuromuscular junctions in insects take up glutamate (Faeder and Salpeter, 1970; Faeder et al., 1974). The nerve branches imaged by the C-terminal antibody run alongside the trachea in some instances, or pass over or under them. In some preparations, the nerves were seen to run the length of the muscle, giving off projections along the muscle surface. A

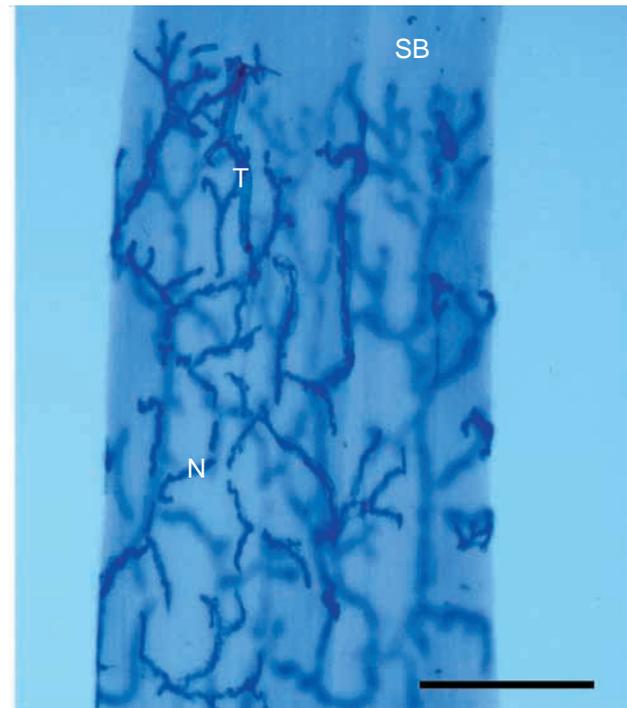


Fig. 4. Isolated larval skeletal muscle fibre stained with anti-C-terminal TrnEAAT1 antibody showing a complex pattern of neuronal innervation. A portion of trachea (T) is filled with air, with axon branches running over and under it. The semi-transparent nature of the preparation reveals that the branching continues around the entire surface of the muscle fibre. The fibre was stained with the alkaline phosphatase substrate Vector Blue. N, motor neuron; SB, segmental boundary. Bar, 500  $\mu$ m.

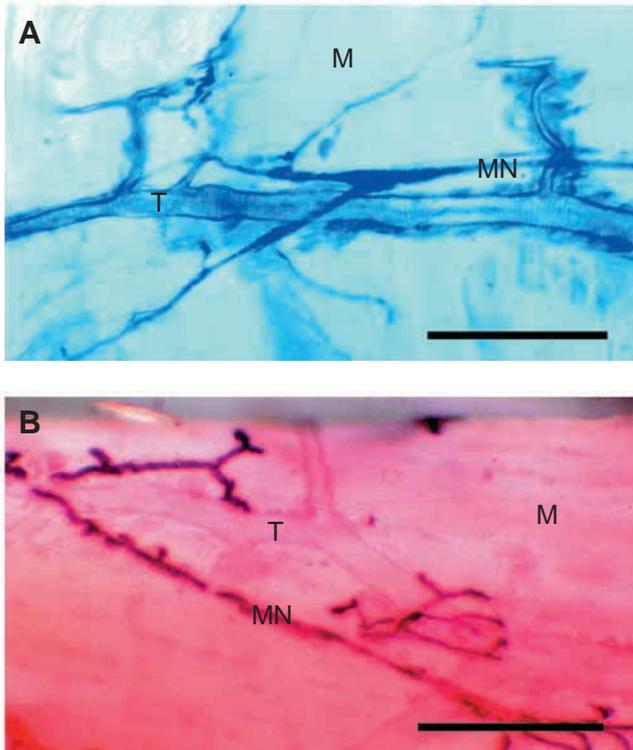


Fig. 5. (A) Methylene Blue stained preparation showing the pattern of nerve innervation on a portion of caterpillar skeletal muscle. (B) Portion of larval muscle, probed with anti-C-terminal antibody and Vector Red staining, showing a motor neuron running alongside and passing under a tracheal segment on the muscle surface. Note the similarity to the Methylene Blue preparation. A, axon; M, muscle; MN, motor neuron; T, trachea. Bar, 100  $\mu$ m.

similar pattern was reported in the larva of the waxmoth *Galleria mellonella* by Belton (1969). *Intra vitam* Methylene Blue staining was used to compare the antibody labelling pattern with that of previously published patterns of nerve distribution on skeletal muscle. The pattern of staining produced by Methylene Blue (Fig. 5A) closely matched the immunohistochemical staining pattern (Fig. 5B). Breaks in the pattern occur along the nerve indicating areas where TrnEAAT1 expression is either obscured by trachea or sites where it is not expressed. The TrnEAAT1 antibodies also stained nerves associated with adult flight- and inter-segmental muscle (not shown). The fine skeletal muscle bundles that run between segments in the adult abdomen exhibited a pattern of innervation similar to that in the caterpillar.

On examining the larval motor neuron terminals at higher magnification, distinctive features of the antibody labelling pattern became evident (Fig. 6). The individual neurons did not appear to be labeled by antibody, but instead their axons were silhouetted as unstained channels surrounded by strongly immunoreactive glial cells. The inner surface of the glial sheath (immediately adjacent to the axons) stained most intensely. Small circular dark plaques, thought to be the sites of the neuromuscular synapses, were distributed along the

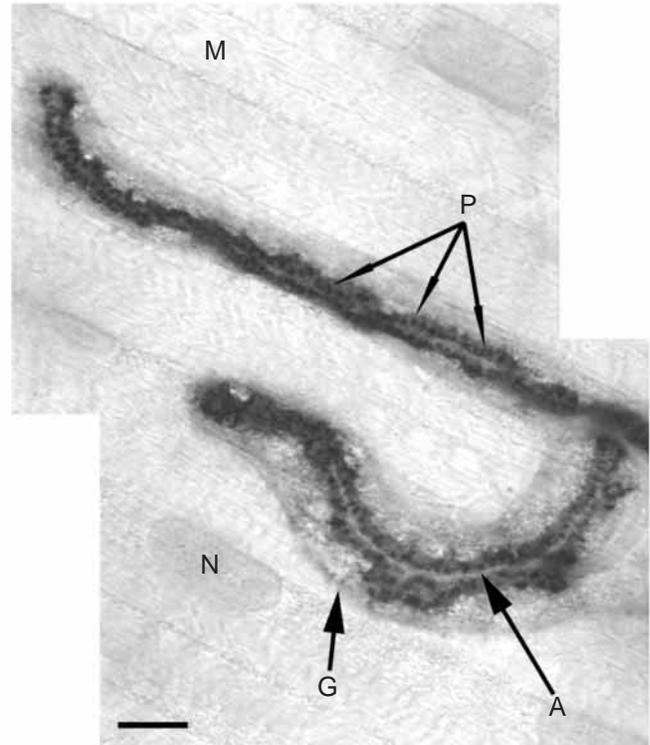


Fig. 6. High magnification view of the surface of a muscle fibre showing an unstained axon that consequently appears light in density, surrounded by heavily stained glial cells. Darkly stained plaques can be seen along the axon. The outer perimeter (arrow, G) of the glial cells is lightly stained. The stain is Vector Red. A, axon; M, muscle fibre; N, muscle nucleus; P, plaque. Bar, 10  $\mu$ m.

tracts formed by the axons and associated glial cells. The outer surface of the glial sheath, as well as the glial cytoplasm was only weakly immunopositive, staining at an intensity barely detectable over the background (muscle) staining (Fig. 6). Except where they form neuromuscular synapses, axon terminals traversing the surface of muscle fibres are wrapped in an immunopositive glial cell sheath. This was seen in cross-sections of muscle in which the transporter had been labelled with a secondary antibody linked to gold and intensified with silver (Fig. 7, inset). All the antibody-labelled glial components are restricted to the outer surface of the muscle, penetrating only a short distance into it. In some cases, glial cells were wrapped over axons that lay in grooves on the muscle surface.

Electron microscopy was used to determine on which membrane surface the glial cells and/or axons expressed EAAT1. Labelling was seen to occur at regular areas over the surface of the muscle in thick sections enhanced with silver (Fig. 7, inset). When viewed in the electron microscope, abundant gold label was seen on the inner surface of the glial plasma membranes (Fig. 7). Labelling occurred only at or near neuromuscular junctions, as seen in Fig. 7 by the presence of a rete synapticum, a specialized membranous network in the

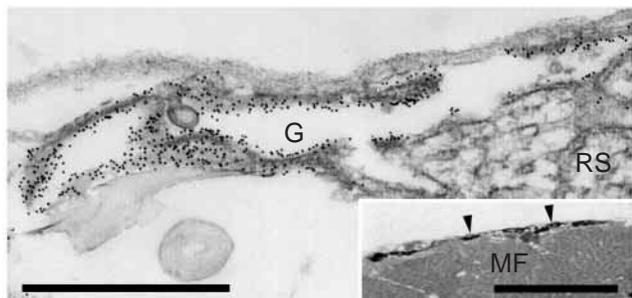


Fig. 7. Electron micrograph of a portion of a glial cell on the surface of a muscle fibre following TrnEAAT1 immunogold labelling. The gold particles are on the cytoplasmic side of the cell membrane where both the N- and C-terminal regions of the transporter are located. G, glial cell; RS, rete synapticum. Bar, 1  $\mu$ m. (Inset) Cross-section (light micrograph) through a muscle fibre showing darkly stained glial cells (arrowheads) that insulate the axons (unstained) from the haemolymph (note similarity to electron micrograph). Tissue was probed with the glutamate transporter C-terminal antibody and visualized with secondary antibody-gold enhanced with silver (tissue stained with Toluidine Blue). MF, muscle fibre. Bar, 100  $\mu$ m.

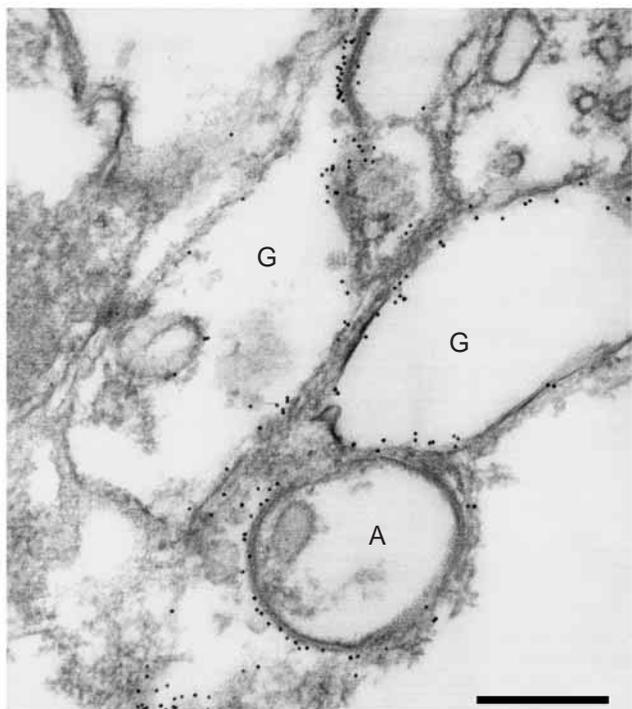


Fig. 8. Electron micrograph of an axon associated with glial cells on a muscle fibre. The glial cell membranes are gold-labelled on cytoplasmic faces. The axon plasma membrane is unlabelled. A, axon; G, glial cell. Bar, 1  $\mu$ m.

muscle that lies between the synapse and the contractile elements of the muscle (Edwards et al., 1958). Fig. 8 shows a glial cell expressing transporter protein on the cytoplasmic face of its plasma membrane where it envelops an adjacent axon. The axonal membrane appears to be unlabelled.

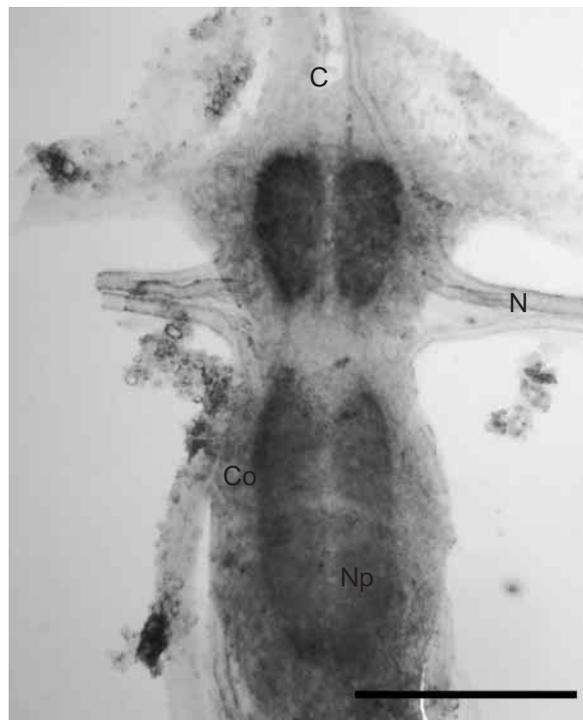


Fig. 9. A caterpillar abdominal ganglion showing dense staining of the inner neuropile (Np). The pattern produced was of a diffuse staining with no cell bodies evident. No staining is evident in the connectives (C), cortex (Co), or nerve branches (N). (BCIP/NBT staining). Bar, 100  $\mu$ m.

#### Nervous system staining

The neuropiles of the ganglia in the larval CNS stained strongly when probed with TrnEAAT1 C-terminal antibody (Fig. 9). The cortex of the ganglion, in which the neuronal cell bodies reside, and the perineurial sheath did not react with the antibody. The neuropile region is composed of finely interwoven nerve terminals interspersed with glial processes. Because synaptic contacts are largely restricted to the ganglionic neuropile in the CNS, a need for glutamate removal from the extraneuronal space in this region might be presumed. The nerve connectives between the ventral ganglia and the nerves branching from them were not stained by TrnEAAT1 antibody. The pattern of EAAT staining was quite different from that seen when anti-FMRamide antibody was used as a control probe for the ganglion. This antibody labelled only cell bodies in the ganglion.

Ultrastructural examination of the ganglion revealed that the transporter was found in the glial cell membranes that insulate the nerve processes in the neuropile (Fig. 10). Gold particles were distributed randomly along the glial membranes and not clustered. No staining was evident in the perineurium, or the ganglionic cortex. This distribution matches the staining pattern seen in the light microscope after chromogenic labelling (Fig. 9). This cellular pattern of label is in agreement with that seen at the neuromuscular junction, where only the glial cell processes were decorated (Figs 7, 8).

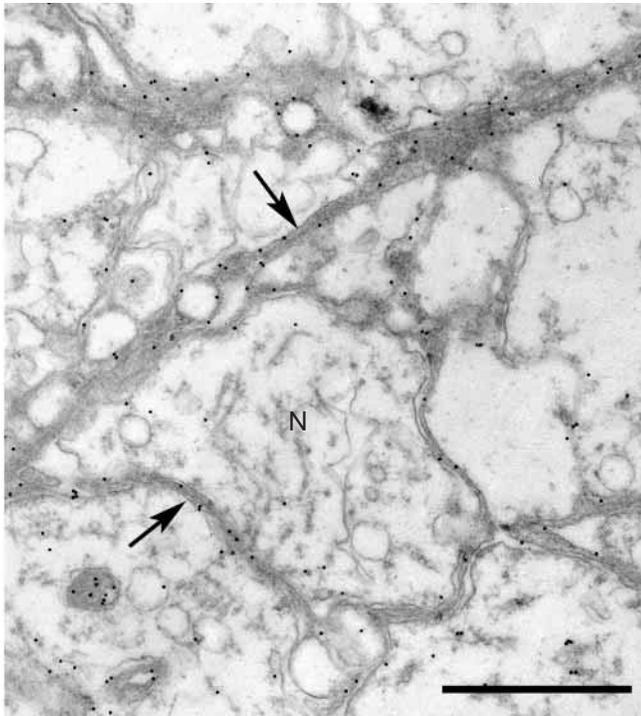


Fig. 10. Electron micrograph of ganglionic neuropile stained with gold-labelled secondary antibody to TrnEAAT1 C-terminal antibody. The glial membranes (arrows) are labeled with gold, with only background labeling evident in nerve cells. N, nerve. Bar, 0.5  $\mu$ m.

### Discussion

We have used immunohistochemical means to localize an EAAT in cabbage looper tissues. Our findings show that the protein is primarily localized to two sites, namely the neuromuscular junctions in skeletal muscle and the neuropile of segmental ganglia (Table 1). This finding does not eliminate the possibility that other types of glutamate transporters exist in insects and remain to be characterized. To date there has been no report of multiple glutamate transporters in a single species of insect, with the possible exception of dEAAT2 in *Drosophila*, which has a selective high affinity for aspartate (Besson et al., 2000).

The distribution of TrnEAAT1 at the neuromuscular synapse, and its specific expression by glial cells confirms earlier physiological studies on glutamate uptake by nervous tissue in insects. Faeder and Salpeter (1970) and Faeder et al. (1974) showed that radioactive glutamate was taken up preferentially by sheath cells surrounding stimulated nerves in a cockroach, with the highest levels of uptake at neuromuscular junctions, rather than in the nerve branches running to the muscle fibres. This observation agrees with the immunohistochemical data reported here. Faeder's observations were later extended to locusts by Botham et al. (1978) and Van Marle et al. (1985), confirming that in insects glutamate is a major neurotransmitter at neuromuscular junctions, and that glial cells play a major role in glutamate recycling. In the caterpillar, only nerves directly in contact with

skeletal muscle and not the nerve fibres running from the ganglion to the muscles stained positively for EAAT. Our data also indicate that the transporter protein is localized to glial membranes and not to axonal membranes. Since axons may travel deep into the muscle and beyond their glial sheaths, it has been proposed that muscle glutamine synthetase may be responsible for glutamate inactivation in areas where glial cells are not present (Dowton et al., 1988). Medium-affinity glutamate transporters are present in epidermal cells in many insects, where they help suppress the levels of glutamate in the haemoplasm (Tomlin et al., 1993). However, local glutamate uptake by glial cells is likely the main regulator of the transmitter glutamate levels at the synapse.

In insects, there are many points of contact between the axons and the muscle fibres. Most insect neuromuscular junctions occur at or near the surface of the muscle (reviewed by Osborne, 1970). This is the case with *T. ni*, where stained nerve processes were seen only running over the surface of the skeletal muscles and not plunging deep within them. In many instances, nerve processes also appear to run along grooves in the muscle fibre. Insect axons are multi-terminal and may make contact with the fibre thousands of times (Osborne, 1975). Judging by the extensive networks of innervating processes that are stained, even on smaller muscles, it would seem that *T. ni* larval skeletal muscles also exhibit this attribute. In our preparations, numerous circular darkly staining bodies are seen running alongside the axon in the glial sheath. We interpret these to be areas of high expression of the transporter by the glial cells at individual neuromuscular synapses. A similar pattern has been shown in the hornworm *Manduca sexta* (Rheuben and Reese, 1978; Rheuben, 1985). Rheuben described circular profiles of glial processes that alternate with muscle extensions contacting the nerve where synapses occur. The insect glutamate transporter appears to be most highly expressed in glial processes between these synapses. The synaptic distribution of this protein in glial cells agrees with findings on some mammalian glutamate transporters. The absence of overall labelling of the glial cell surface indicates that following its synthesis the transporter protein is rapidly directed to the cell surface nearest sites of transmitter release. The mammalian GABA transporter, is also known to be targeted to sub-domains of the cell surface (Pietrini et al., 1994).

Considering that the caterpillar glutamate transporter was cloned from a head cDNA library, it is hardly surprising that the transporter was subsequently found to be expressed in the CNS of this insect. TrnEAAT1 was detected in the central neuropile only, and not in the connectives or nerve branches that communicate from the ganglion to other parts of the insect's body. Individual glial cell bodies were not stained as was seen when FMRFamide was localized in this insect's CNS (R.B.G., unpublished). Instead a diffuse glial distribution in the neuropile was demonstrated. The neuropile contains numerous axonal connections that require the removal of glutamate after signaling occurs. This is not the case in connectives and communicating nerves, which form synapses only when they terminate at a muscle or another nerve(s). Our findings on the

localization of the transporter in the insect CNS are consistent with the reported localization of glutamate transporters in the mammalian CNS (for a review, see Danbolt, 2001). Although some of these transporters are expressed by neurons (EAAT3), the transporters most important for glutamate removal are expressed by glial cells (EAAT-1 and -2).

This is the first description of the cellular pattern of localization of a glutamate transporter protein at the neuromuscular junction and in the CNS of an insect. It will prove interesting to see whether other glutamate transporters are localized in a similar fashion in excitable tissues of other insects. Currently we are examining other insect transporters to determine their localization and co-expression in glial or neuronal cells. These studies should help delineate the roles of these essential proteins in synaptic signaling in insects.

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