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Gamma-Aminobutyric Acid in the Nervous System of a Planarian

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ABSTRACT

The amino acid gamma-aminobutyric acid (GABA) is an important inhibitory neurotransmitter in both vertebrates and invertebrates. Despite this, no reports of GABA in flatworms have to date been published. We have studied the presence of GABA in the planarian *Dugesia tigrina* with immunocytochemical methods and high-pressure liquid chromatography. Fibers showing GABA-like immunoreactivity (GABA-IR) were present in abundance in the longitudinal nerve cords and lateral nerves. GABA-IR was revealed in fibers forming commissures in the brain. The ventral part of the subepidermal plexus showed GABA-IR. No cell somata containing GABA-IR could be identified with certainty.

The chromatographic analysis showed that the average GABA concentration in *D. tigrina* is 533.6 pmol/mg protein. This is substantially higher than the concentrations of dopamine (62.87 pmol/mg) and serotonin (233.20 pmol/mg). An enzyme assay confirmed the capacity for GABA-synthesis in *D. tigrina*. The results indicate that GABA-containing neurons appeared earlier in evolution than was previously thought and that GABA may serve an important role already in the flatworms.

Key words: platyhelminthes, *Dugesia*, HPLC, immunocytochemistry, amino acids

The amino acid gamma-aminobutyric acid (GABA) is the most important inhibitory interneuronal transmitter in vertebrates and is present in high concentrations in the brain (Roberts, 1986). In invertebrates, it is an interneuronal transmitter in all studied phyla, and it is an inhibitory neuromuscular transmitter in several phyla (Walker and Holden-Dye, 1991). In the nematodes *Ascaris suum* and *Caenorhabditis elegans*, it is present in inhibitory motoneurons (Johnson and Stretton, 1987; McIntire et al., 1993), whereas it is restricted to the central nervous system (CNS) in the molluscs *Limax maximus* and *Macoma balthica* (Cooke and Gelperin, 1988; Karhunen et al., 1993). In crustaceans, it is present in inhibitory motoneurons in the legs and in interneurons in the thoracic ganglion (Homberg et al., 1993). In insects, GABA is present in the CNS and inhibitory motoneurons (Watson, 1986), and there is evidence that GABA is the inhibitory transmitter in insect neuromuscular junctions (Usherwood and Grundfest, 1965).

Despite this widespread occurrence of GABA in the animal kingdom, no reports of a presence of GABA in flatworms have to date been published. In marine polyclad flatworms, treatment with GABA or glycine at concentrations as low as 1 μM decreases the electrical activity in the ventral nerve cords. This inhibitory effect is reversible and can be prevented with picrotoxin, bicuculline, or strychnine (Keenan et al., 1979). This suggests that receptors that bind GABA and glycine are present in flatworms.

There have been several reports of invertebrate ion channel receptors that are rather nonspecific with respect to the ligand. In crustaceans and molluscs, chloride channels that are activated by GABA, glutamate, and acetylcholine have been described (King and Carpenter, 1987; Hatt and Franke, 1989). This indicates that a pharmacological response to GABA in an invertebrate preparation does not necessarily imply the presence of GABA. It has been generally assumed that flatworms do not utilize GABA (see Raether, 1988; Pax and Bennett, 1991). In an attempt to ascertain whether the known actions of GABA in flatworms are reflected by the actual presence of the substance, we have studied the localization and concentration of GABA in the planarian *Dugesia tigrina*. We also measured the rate of GABA synthesis by glutamate decarboxylation.

The morphology of the nervous system in planarians of the genus *Dugesia* has been rather thoroughly described by Baguna and Ballester (1978). The following section is based mainly on their description.

The CNS is made up of a bilobed brain in the anterior end of the animal and a pair of ventrally located longitudinal nerve cords (LNC) that decrease in diameter as they continue caudally. The LNC are connected to each other by serially arranged commissures throughout their length.

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From the LNC, numerous branches run in a lateral direction and feed into peripheral plexuses. These lateral nerves and the LNC extend in a single plane. Each of the LNC consists of fiber bundles that run in parallel and only occasionally make contact. At regular intervals, there are points with extensive branching; these are rich in synaptic contacts. The brain contains many fibers that connect the two halves. There is no sheath or capsule to demarcate the CNS from the parenchyme; it has a rather spongy structure and is interspersed with lacunae. These lacunae contain both neural and nonneural cell somata as well as muscle fibers and cytoplasmic processes from parenchymal cells. This arrangement may provide a means to transport nutrients and metabolites in this animal, which lacks a circulatory system, other than the gut.

The most prominent peripheral plexus is the submuscular plexus, a regular network of fiber bundles that innervate the muscular body wall. This plexus also sends fiber bundles to the CNS at the level of the LNC. A subepidermal plexus, consisting of single, thin (0.1–0.2 μm) fibers, extends in a single plane under the basal membrane of the epidermis. This plexus is believed to perform both sensory and motor functions. Thin fibers connect the submuscular and subepidermal plexi. The pharynx is innervated by an inner and an outer plexus and the intestinal branches are surrounded by a gastrodermal plexus.

Welsh and Williams (1970) described cell somata that appeared to contain, in formaldehyde-treated planarians, both catecholamines (CA) and serotonin (5-HT). They found cells that were mono-, bi-, or multipolar in shape. The smallest cell somata could be distinguished from axonal varicosities only if their nuclei were visible. In the brain the authors found a few large (15–20 μm) and many smaller CA-containing cell somata. In the case of *D. tigrina*, the large cells were restricted to two pairs. The largest (10–15 μm) of the 5-HT cells were located posteriorly to the brain, whereas many smaller ones were spread throughout the nervous system. CA- and 5-HT-containing cells were rather evenly distributed along the LNC, with aggregations of 5-HT cells at the branching points of the LNC. The neuropil of the brain showed CA-like fluorescence, and the LNC contained fibers with both CA and 5-HT. In the submuscular and subepidermal plexi, the dorsal parts contained mainly CA, while the ventral parts appeared to contain much more 5-HT (Welsh and Williams, 1970). Kabotyanski and colleagues (1991) have shown that the submuscular plexus exhibits immunoreactivity (IR) for 5-HT and appears to send 5-HT-containing fibers towards the ventral epithelium and that the pharynx is richly innervated with 5-HT-immunoreactive fibers.

The presence of dopamine (DA), norepinephrine (NE), and 5-HT in *Dugesia* has been confirmed with chromatographic methods by Welsh (1972), and catecholamines seem to be very abundant among planarians (Eriksson et al., 1993). A photoperiodically modulated melatonin content has been described in *Dugesia* (Morita et al., 1987). Antisera raised against at least 13 neuroactive peptides show IR in flatworms (for review, see Gustafsson and Reuter, 1992), and recently the first two native flatworm neuropeptides, neuropeptide F and GNFFRFamide, were isolated and sequenced (Maule et al., 1991, 1993).

At present, there is strong evidence for an actual neurotransmitter role in planarians for 5-HT and DA only. 5-HT has excitatory effects on the pharynx and on the ciliated cells of the ventral surface, and it also has a general activating effect on planarian metabolism (Webb, 1988; Kabotyanski et al., 1991). Several drugs that affect DA receptors alter the motor activity in planarians of the genus *Dugesia* (Venturini et al., 1989).

MATERIALS AND METHODS

Animals

The planarians were from a several-years-old laboratory culture of an asexual strain of *D. tigrina* (Girard) that had been kept in tap water and fed weekly with raw beef liver. Starved specimens ranging from about 4 to 12 mm in length were used.

Fixation

To get the worms as flat as possible in the fixed state, they were put on a damp filter paper in a 5 cm petri dish kept on ice prior to fixation. Only about 500 μl of cold fixative was added in the beginning. This amount saturated the filter paper and enabled the fixative to penetrate from the ventral side while the worms were unable to move or curl. When the worms appeared to be dead, usually after about 10 minutes, they were immersed in fixative.

The fixatives were either 4% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC; Sigma, St. Louis, MO) and 2% formaldehyde (FA; Merck) in 0.1 M phosphate buffer, pH 7.4, with 0.25% Triton X-100 (PBS-T) for 3–12 hours at 4°C (Panula et al., 1988) or 4% FA and 1% glutaraldehyde (GA; Merck) in PBS-T for 2–3 hours in 4°C. After fixation, the worms were rinsed in PBS and kept in 20% sucrose in PBS in 4°C for at least 3 hours. They were then cut on a cryostat into 20-μm-thick sections that were collected on coated glass slides. The EDAC/FA-fixed sections were treated with 0.5% sodium borohydride (Merck) for 30 minutes at room temperature to reduce free aldehydes that could cause excess background staining (Airaksinen et al., 1992).

Antisera and controls

Most of the staining procedures were performed with an anti-GABA antiserum (1H) raised in rabbits against GABA conjugated with EDAC to the carrier protein keyhole limpet hemocyanin (Airaksinen and Panula, 1990). Karhunen et al. (1993) have shown that this antiserum has a high specificity for GABA conjugates. Controls for the specificity of the antiserum for GABA were done by incubation of the diluted antiserum with EDAC conjugates of GABA with ovalbumin (1 and 5 μg/ml) and of glutamate with bovine serum albumin (50 μg/ml).

To verify the results, a monoclonal mouse anti-GABA antibody (mAb) was used. This mAb was provided by Prof. I. Virtanen (University of Helsinki). The specificity of the mAb (115AD5) for FA/GA-linked GABA-protein conjugates has been thoroughly described (Szabat et al., 1992). The mAb was applied to FA/GA-fixed sections. Attempts to stain EDAC/FA- and FA-fixed sections were also done.

Immunocytochemistry

The rabbit anti-GABA antiserum was diluted 1:500 in PBS-T with 1% normal swine serum and applied overnight to the EDCDI/FA-fixed sections at 4°C. The mAb was diluted 1:10 in PBS-T with 1% NSS and applied to the FA/GA-fixed sections either overnight at 4°C or for 2 hours at room temperature. After the incubation with anti-GABA

serum or mAb, the slides were washed in three changes of PBS-T for a total of 45 minutes.

The procedure continued with the indirect immunofluorescence method (Coons, 1958). The secondary antisera were either fluorescein-labelled swine antirabbit IgG (DAKO) for the anti-GABA serum or fluorescein-labelled rabbit antimouse IgG (DAKO) for the mAb. In both cases, the secondary antiserum was diluted 1:40 in 1% normal swine serum and applied for 45–60 minutes at room temperature. After three washes totalling 45 minutes, 50% glycerol in PBS was placed on the slides and a coverslip was applied.

High-pressure liquid chromatography

To verify the presence of GABA in *D. tigrina*, reverse-phase high-pressure liquid chromatography (RP-HPLC) with fluorescence detection was performed. This was done according to the method described by Tamura et al. (1990), with minor modifications. Briefly, the amino acids were derivatized with 10 mM *o*-phthalaldehyde (OPA; Fluka) in 0.4 M sodium borate buffer (pH 10.2) in the presence of 4.5 μl/ml 2-mercaptoethanol (Sigma) for 2 minutes at 12°C. The OPA derivatives of amino acids were separated on a TSKgel ODS-80 TM (150 × 4.6 mm, 5 μm; Tosoh) reverse-phase column in two different 30 minute nonlinear gradients from 25% to 80% methanol in 0.1 M potassium acetate buffer, pH 5.1, at a flow rate of 0.8–1.0 ml/minute. The excitation wavelength was 360 nm, and the fluorescence was measured at 455 nm.

The RP-HPLC equipment comprised a CMA/200 programmable autoinjector that derivatized and injected the samples, a Pharmacia LKB 2150 HPLC pump, an LKB 2152 HPLC controller with an LKB gradient mixing valve and an LKB Ultrograd mixer driver, a Hitachi F-1050 fluorescence spectrophotometer, and a Hitachi D-2500 chromatointegrator.

The concentrations of NE, DA, and 5-HT were measured with RP-HPLC with electrochemical detection. The apparatus and chromatographic conditions for measurement of DA and NE were the same as described by Eriksson et al. (1993). The tissue was homogenized in 0.4 M perchloric acid (PCA; Merck) containing 3,4-dihydroxybenzylamine as internal standard, and the catechol derivatives were purified on alumina at pH 8.6 according to the method of Eriksson and Persson (1982). After elution of the bound catecholamines with 2% acetic acid, the separation was done on a Nova-Pak column (150 × 3.9 mm, 4 μm; Waters) in a mobile phase composed of 3% acetonitrile and 97% of a buffer containing 0.05 M citric acid monohydrate, 0.05 M potassium phosphate, 55 μM EDTA (all from Merck), and 1.2 mM octanesulfonic acid (Sigma), pH 2.80, at a flow rate of 1.2 ml/minute. 5-HT measurements were carried out by homogenization in 0.4 M PCA and direct injection of the centrifuged homogenate into a mobile phase composed of 13% acetonitrile in the same buffer and flow rate on the same column. The RP-HPLC measurements of GABA, DA, NE, and 5-HT were all done in triplicate, and each sample consisted of three to five planarians.

Glutamate decarboxylase assay

Due to a shortage of animals, only two samples, A and B, each containing three planarians, were homogenized in 300 μl of buffer. The homogenization buffer for sample A was composed of 20 μM pyridoxal-5-phosphate (PLP; Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), 2 mM EDTA (Merck), 1 mM reduced glutathione (Merck), and 1% poly-

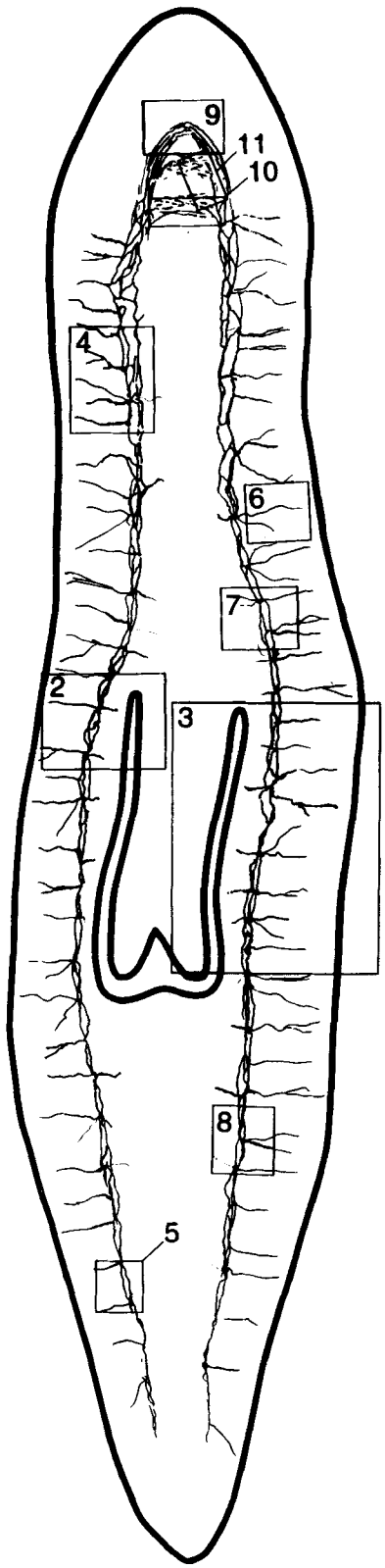
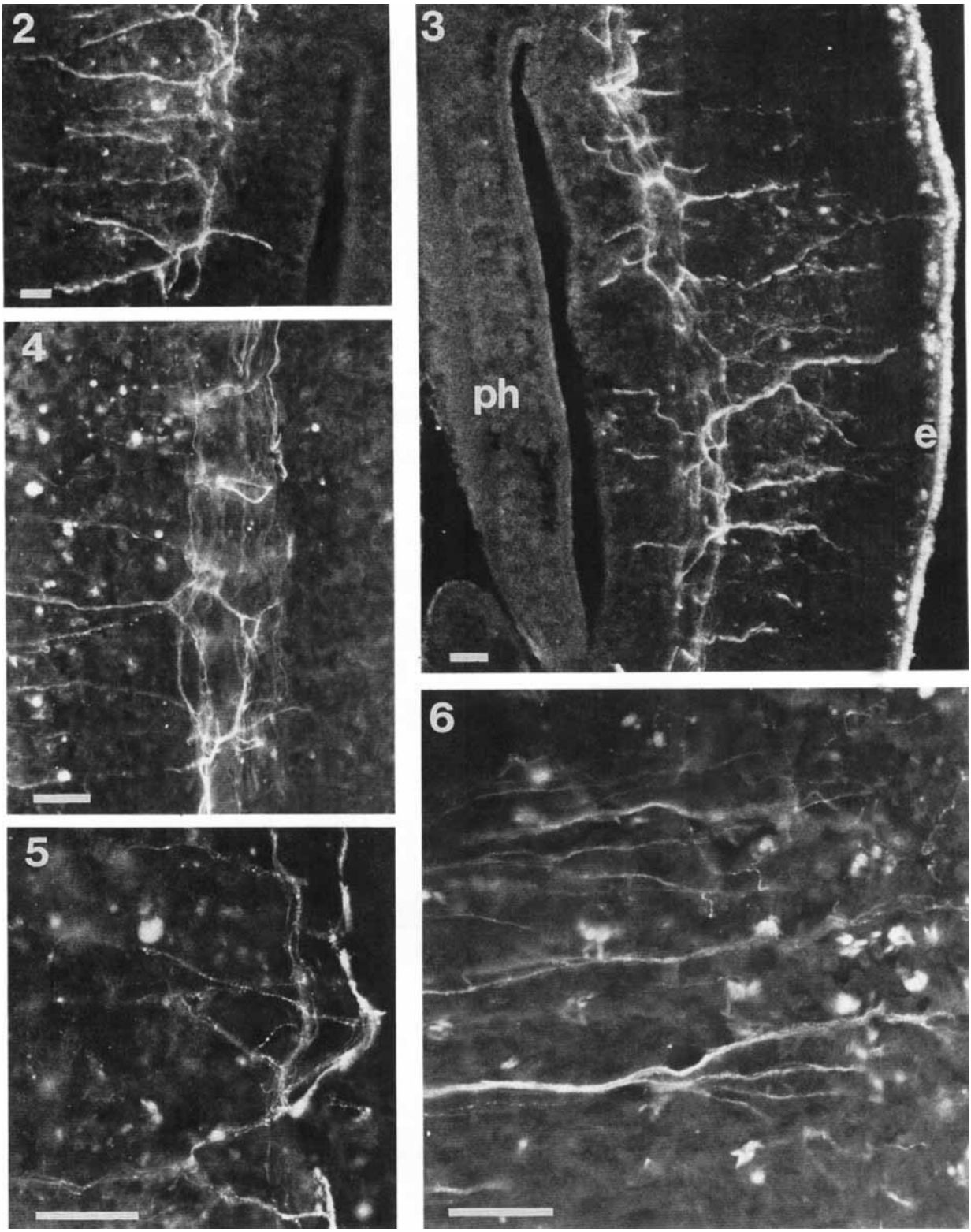


Fig. 1. An overview of the typical appearance of the GABA-IR in the brain, in the two longitudinal nerves, and in the numerous lateral nerves in *D. tigrina*. The body margin and the pharynx are indicated with a wider line. The GABA-IR in the more ventrally located subepidermal plexus is left out from the drawing for the sake of clarity. The approximate locations of Figures 2–11 are indicated in the drawing. Animals of different sizes have been used, and Figures 2–11 are therefore not all to scale with Figure 1.



Figs. 2–6. **Figures 2** and **3** show GABA-IR in the longitudinal nerve cords (LNC) on each side of the pharynx. Long lateral nerves run towards the body margin, and shorter processes extend towards the tube-shaped pharynx. To the left in Figure 3, a cut through all tissue layers of the pharynx (ph) shows that no GABA-IR is present in any part of the pharynx. To the right in Figure 3, the highly autofluorescent epidermis (e) can be seen. In **Figure 4**, a stretch of LNC from the anterior part of the animal shows commissure-like structures inside the nerve cord. In this part of the animal, the LNC usually exceed 100 μm in width. Thin lateral nerves running to the left can also be seen. In **Figure 5**, a stretch of posterior LNC is shown. The width of this part of the LNC is 20–30 μm. **Figure 6** shows the area lateral to the right LNC. A branching nerve and thin fiber bundles as well as single fibers can be seen. An irregular pattern of subepidermal fibers can be seen in the upper right half of the picture. Scale bar = 50 μm.

ethylene glycol (MW 300; Sigma) in 0.1 M potassium phosphate buffer (pH 6.8). Sample B was homogenized in a buffer with the same composition, but without the coenzyme PLP. The homogenates were centrifuged at 10,000*g* for 15 minutes, and the small molecules in the supernatants were removed with centrifuge filters (Ultrafree-MC TTK; Millipore) that have a nominal molecular weight limit of 30,000 daltons, according to the manufacturer.

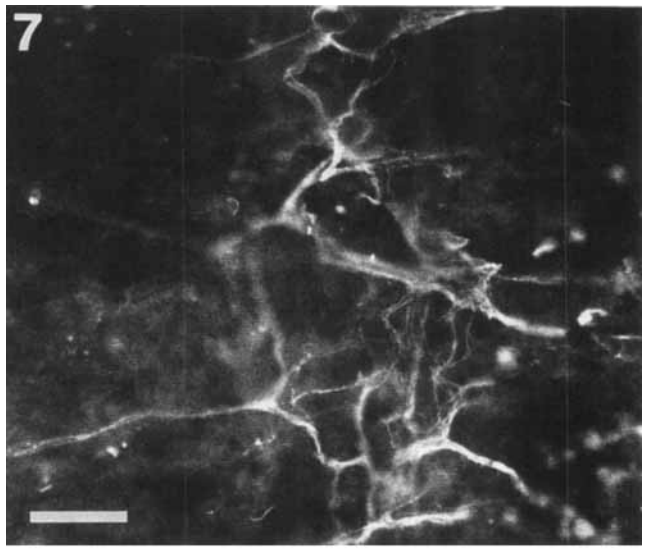
Sample A was resuspended in 90 μl of PLP-containing buffer, and 10 μl of 10 mM glutamate was added. This was divided into two 50 μl portions (samples 0 and 1), one of which (sample 0) was promptly treated with 5 μl of 4 M PCA. Sample B was suspended in 90 μl PLP-lacking buffer, 10 μl of 10 mM glutamate was added and divided into two 50 μl portions (samples 2 and 3), and one of these (sample 3) was made to 20 μM PLP by addition of 1 μl of 1 mM PLP. All steps were performed on ice or at 4°C. After this, the four samples were incubated on a water bath at 30°C for 4 hours. The incubation was terminated with addition of 5 μl of 0.4 M PCA to samples 1–3, and after centrifugation GABA was measured as described above. The GABA content of the glutamate solution was also measured.

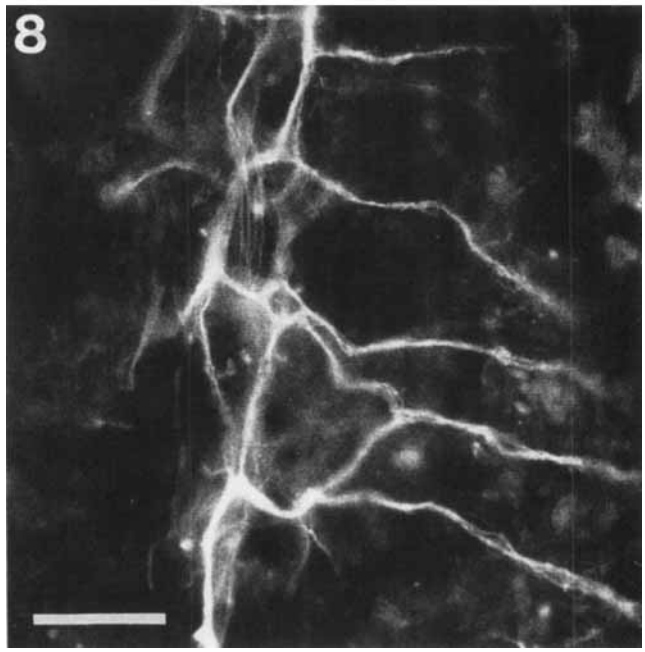
RESULTS

Immunocytochemistry

The antiserum and the mAb both indicated the presence of GABA in *D. tigrina*, and their immunoreactivities were restricted to the same parts of the nervous system. Strong GABA-IR was seen in the longitudinal nerve cords (LNC) throughout the entire length of the animal. GABA-IR was also revealed in the lateral nerves extending from the LNC. The GABA-IR in the horseshoe-shaped brain was distinct but somewhat weaker than in the longitudinal and lateral nerves. The ventral subepidermal nerve plexus also showed IR. Cell somata with GABA-IR could not be identified with confidence. No IR was seen in the pharynx or in the submuscular or gastrodermal plexi. In contrast with the ventral subepidermal plexus, the dorsal subepidermal plexus showed no IR. The mAb gave slightly weaker IR in the nerve bundles and stained fewer fibers than the rabbit antiserum. This difference was barely noticeable in the LNC, but it was more obvious in the lateral nerves. Strongly autofluorescent tissue elements were common, but, because this autofluorescence either was red or yellow or had a green color different from that of fluorescein, it did not affect the identification of IR. However, it did affect the photographs, and all rounded white structures in the pictures are autofluorescent.

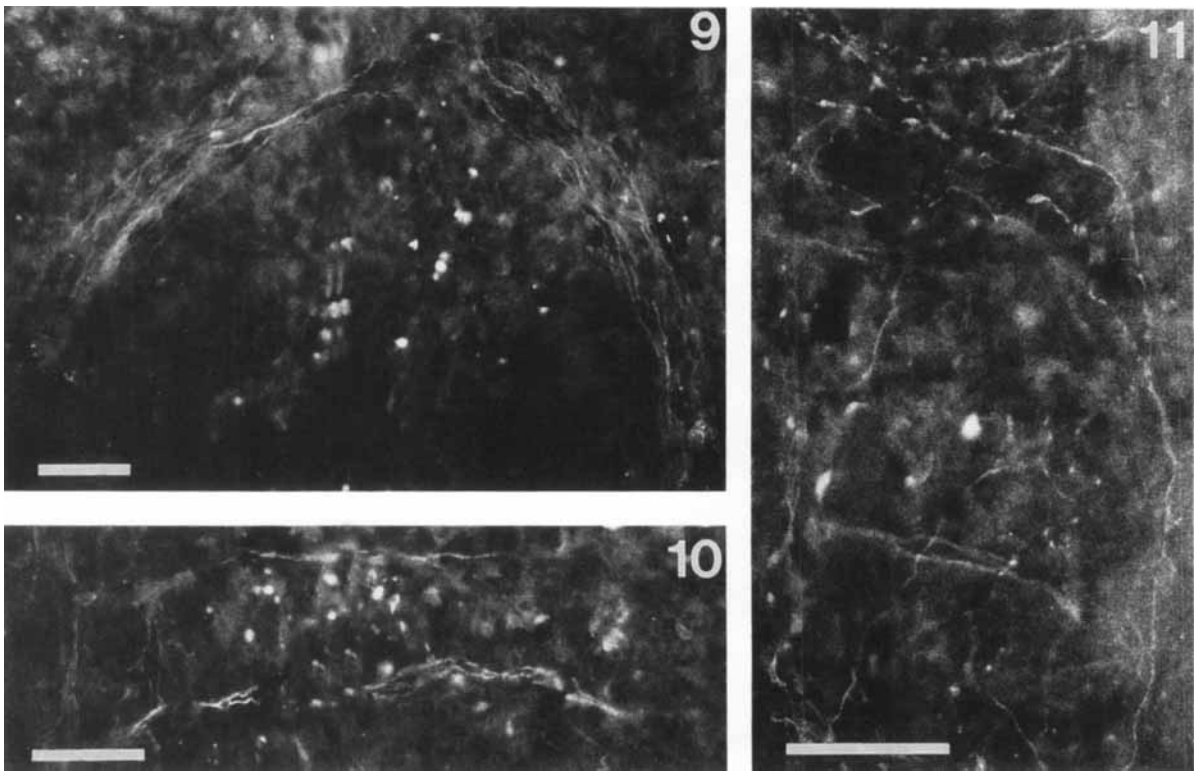
Figure 1 gives an overview of the GABA-IR in nervous structures in *D. tigrina*. The body margin and the pharynx are indicated by wide lines. The rest of the drawing shows the GABA-IR of nerve structures in a frontal section cut at the level of the LNC and lateral nerves. These nerves extend in the same plane, while the ventral subepidermal plexus lies at a deeper level. The plexus is left out to prevent the drawing from becoming too complicated. The approximate locations of Figures 2–11 are indicated in the drawing. Animals of different size were used, and Figures 2–11 are therefore not all exactly to the same scale as Figure 1. Figure 1 is based on immunostaining of different parts of several planarians. It should not be regarded as an exact description of a particular specimen, since the locations of individual nerves vary from animal to animal.





Figs. 7, 8. Typical appearance of the LNC, with GABA-IR fibers running both in bundles and alone. In both figures, lateral nerves that run to the right are demonstrated. In Figure 7, a bundle of several fibers runs towards the center of the animal. Scale bar = 50 μm.

Figures 2 and 3 show GABA-IR in the LNC on each side of the pharynx (ph). Long branches run toward the body margin, and shorter processes extend toward the pharynx. Branching from the LNC toward the center of the animal is more abundant close to the pharynx than along the rest of the LNC. Despite this, no innervation of the pharynx could be seen. To the right in Figure 3, the highly autofluorescent epidermis (e) can be seen. The LNC can exceed 100 μm in width in the anterior part of the animal (Fig. 4) and decrease to well under 50 μm caudally (Fig. 5). Most immunoreactive fibers that leave the LNC run in the lateral nerves, but single fibers also leave the LNC (Fig. 5). The lateral nerves divide into thinner branches as they continue



Figs. 9–11. GABA-R in the brain of *D. tigrina*. No cell somata can be safely identified, but several strongly autofluorescent dots are present. In **Figure 9**, broad streaks of single fibers that run from each of the LNC and meet in the anterior end of the brain are shown. **Figure 10** shows commissures that connect the LNC in the posterior part of the brain. **Figure 11** shows an irregular pattern of single fibers in the center of the brain. Scale bar = 50 μm.

laterally. In Figure 6, the lateral direction is to the right, and a branching nerve and thin fiber bundles as well as single fibers can be seen. An irregular pattern of subepidermal fibers can be seen in the upper right half of the picture. The LNC are highly branched and show an irregular pattern (Figs. 7, 8). Fibers from the LNC continue into the brain and meet in the anterior part of the brain (Fig. 9). In the posterior part of the brain, immunoreactive fibers form commissures between the two LNC (Fig. 10). An irregular pattern of fibers can be seen between the two halves in the area between the commissures and the frontal end of the brain (Fig. 11). The brain is the only location where GABA-IR has been detected in connections between the LNC. Most of the strongly fluorescent dots in Figures 9–11 are autofluorescent, and no immunoreactive cell somata can be safely identified. The ventral subepidermal plexus shows GABA-IR, and the fishnet-like pattern in this plexus is seen in Figures 12–14. Because the epidermis exhibits strong autofluorescence, the plexus is difficult to study. Autofluorescent structures are ubiquitous among the thin immunoreactive fibers.

Controls for specificity

Preincubation with 5 μg GABA-ovalbumin conjugate per milliliter of diluted antiserum abolished all IR, whereas 1 μg caused no detectable decrease in the IR. Fifty micrograms of glutamate conjugate decreased the IR only very slightly (Fig. 15). Attempts to stain EDAC/FA- and FA-fixed sections with the mAb gave no IR.

HPLC and glutamate decarboxylase assay

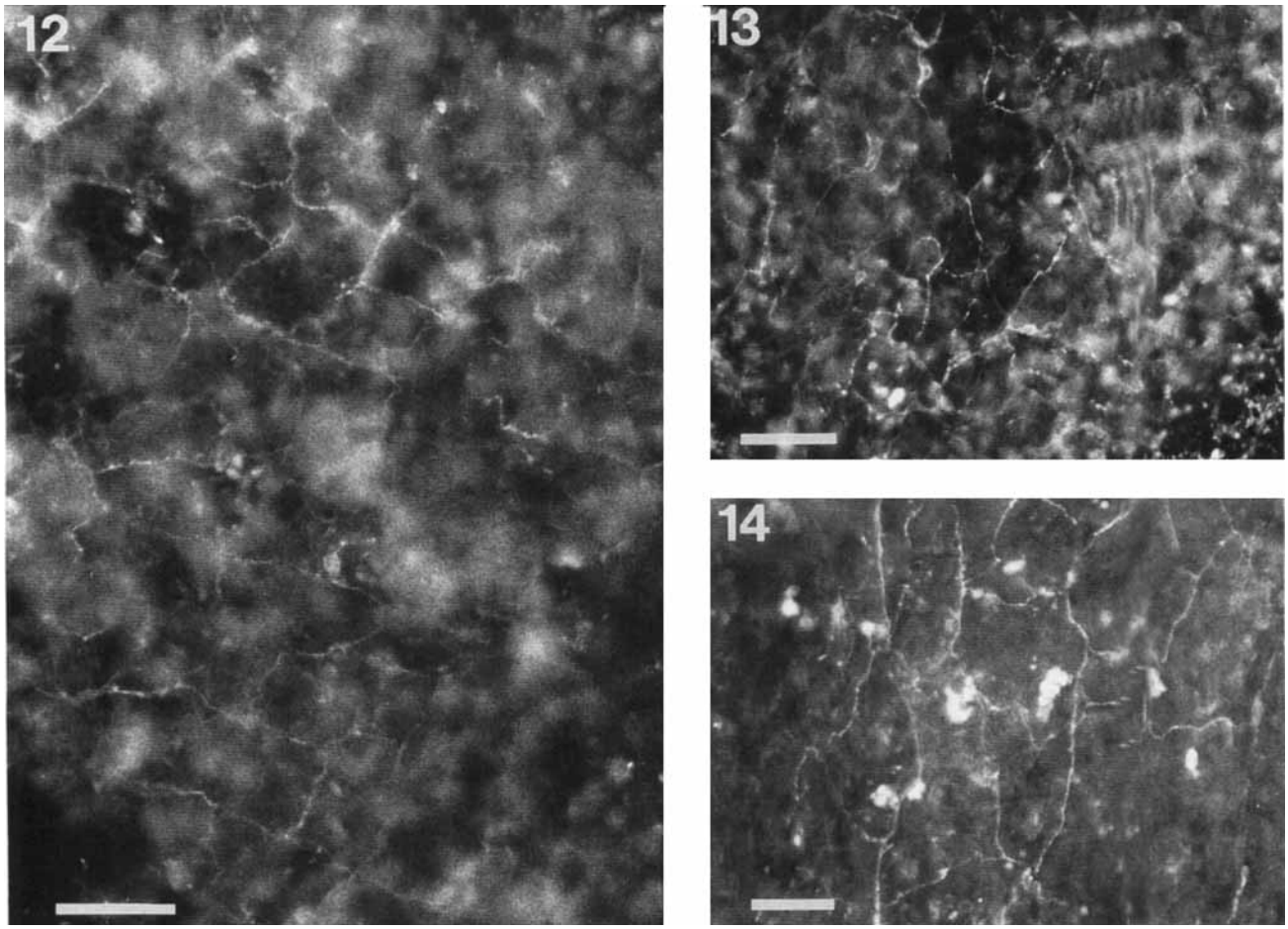
The RP-HPLC analysis verified the presence of GABA in *D. tigrina*. Table 1 shows the concentrations of GABA, 5-HT, DA, and NE in the animal. The concentration of GABA is more than twice that of 5-HT and more than eight times higher than that of DA.

The samples 1 and 3 from the enzyme assay contained 4.10 and 3.60 nmol GABA/mg protein after incubation, respectively. Sample 2, which was incubated without the coenzyme PLP, contained 1.54 nmol/mg. Sample 0, which was inactivated before the incubation, contained 0.28 nmol/mg. The glutamate used as substrate contained a GABA contamination of 0.02% that has been subtracted from these values.

DISCUSSION

There has been only one previous report of a putative amino acid transmitter in a flatworm. Webb and Eklove (1989) reported glutamate-like IR in the LNC of the tapeworm *Hymenolepis diminuta*. Glutamate and GABA show structural similarities, and we performed preabsorption tests with glutamate conjugate to exclude the possibility of a cross-reaction with glutamate.

The antiserum stained more fibers than the monoclonal antibody in the lateral nerves. The reason for this is not clear, but because the difference was more prominent in the thinnest fiber bundles and single fibers, the fixation process



Figs. 12–14. GABA-IR in the thin fibers of the ventral subepidermal plexus. The rather regular, fishnet-like pattern is visible. All rounded structures are autofluorescent. Scale bar = 50 μm.

may be the cause. However, the monoclonal antibody stained the ventral subepidermal plexus and single fibers in the brain, indicating that differences in the concentration of GABA may explain the discrepancy.

We could not confidently identify any cell somata with GABA-IR, indicating that these cells are very small. The small size of most planarian neurons is well known (see Morita and Best, 1966; Welsh and Williams, 1970).

Previous reports on catecholamines and 5-HT in planarians have related the concentrations to wet weight (Welsh, 1972), while our data on DA, NE, and 5-HT are given as picomole per milligram protein. A comparison between earlier data and our present data show a very good concordance, provided that the protein content in fresh planarian tissue is about 8%, which seems reasonable.

There is a higher variability in the concentrations of GABA and 5-HT compared to the catecholamines. This may reflect the actual situation in these animals but may also be due to the fact that an internal standard was used in the DA and NE measurements, while GABA and 5-HT were compared to external standards. An internal standard compensates for both sample loss and concentration due to evaporation during the sample pretreatment and therefore makes more exact measurements possible.

The relative concentrations of different substances do not necessarily correlate with their biological importance, but the high concentration of GABA compared with 5-HT and DA indicates that GABA plays a significant role in the planarians. Both 5-HT and DA have important functions in these animals (see Webb, 1988).

The result from the enzyme assay shows that GABA synthesis can take place in these animals, but the molecular weight filtration did not remove all GABA from the homogenates, so an exact quantification of the enzyme activity remains to be done. However, because samples 0 and 1 contained equal amounts of the same homogenate, the 15-fold increase in GABA must have taken place during the incubation. Samples 2 and 3 contained equal amounts of a divided homogenate lacking the coenzyme PLP. Sample 3 received PLP, and after the incubation it contained more than twice the amount of GABA compared to sample 2. The unexpectedly high concentration of GABA in sample 2 may be due to some endogenous PLP that have remained bound to the enzyme during the filtration. The result indicates at least partial dependence on PLP and that the GABA synthesis in planarians is catalyzed by an enzyme that has similarities with the glutamate decarboxylase of higher animals.

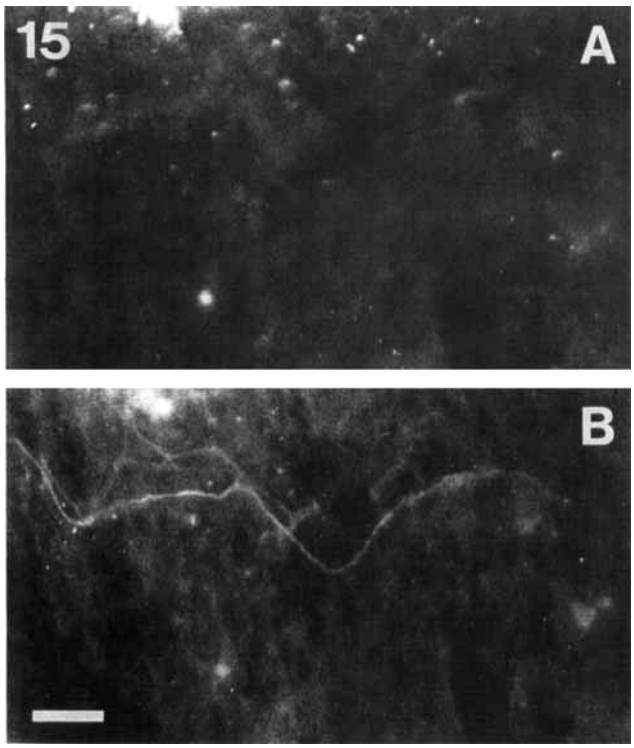


Fig. 15. **A** and **B** show the same section, first (A) stained with anti-GABA serum that had been treated with 5 μg/ml GABA conjugate and thereafter (B) stained with anti-GABA serum treated with 50 μg/ml glutamate conjugate. In A, only the autofluorescence can be seen. Scale bar = 50 μm.

TABLE 1. Concentrations of Putative Neurotransmitters in *Dugesia tigrina* (pmole/mg protein ± S.D.)

GABA	533.60 ± 133.20 (N = 3)
Serotonin	233.20 ± 53.04 (N = 3)
Dopamine	62.87 ± 6.44 (N = 3)
Norepinephrine	11.60 ± 1.47 (N = 3)

It is a common assumption that all the bilaterally symmetrical animals (Bilateria) have evolved from a flatworm-like ancestor (see Barnes et al., 1988). Comparisons of 18S ribosomal RNA-sequences have shown that present-day flatworms have a separate phylogenetic radiation and therefore make up a sister group to the rest of Bilateria (Field et al., 1988; Adoutte and Philippe, 1993). It is most likely that features of the nervous system that present-day flatworms share with the more advanced animals were present already in the ancestral flatworms, although the possibility that a certain characteristic has evolved twice cannot be ruled out. Data on flatworm neurochemistry is therefore of considerable phylogenetic interest.

Our results show that GABA appeared earlier in evolution than was previously thought and that it may serve an important role already in the nervous system of flatworms. The vertebrate $GABA_A$ and glycine receptors are ligand-gated chloride channels that bear striking similarities and are believed to be closely related and to originate from a common ancestor (Barnard et al., 1987). This common ancestral receptor can be expected to have occurred in flatworms or even earlier.

More evidence is needed before GABA can be assigned an actual neurotransmitter function in flatworms. However, the widespread presence of GABA in the nervous system, the comparatively high concentration, and the capacity for synthesis of GABA together with its known inhibitory action that can be blocked by GABA antagonists (Keenan et al., 1979) suggest a function in these animals. We have also done a preliminary immunoelectron microscopic study that has revealed GABA-IR in close association with muscles in the body wall and in cell somata in the LNC. All this suggests that GABA functions as a transmitter, most likely inhibitory, in flatworm neuromuscular synapses.

The study will be expanded to parasitic flatworms, among which the most important are the blood fluke *Schistosoma mansoni* and the liver fluke *Fasciola hepatica*. As is the case with nematode parasites, GABAergic neurotransmission in parasitic flatworms would be a possible target for chemotherapy.

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LITERATURE CITED

Adoutte, A., and H. Philippe (1993) The major lines of metazoan evolution: Summary of traditional evidence and lessons from ribosomal RNA sequence analysis. In Y. Pichon (ed): Comparative Molecular Neurobiology. Basel: Birkhäuser Verlag, pp. 1–30.

Airaksinen, M.S., and P. Panula (1990) Comparative neuroanatomy of the histaminergic system in the brain of the frog *Xenopus laevis*. J. Comp. Neurol. *292:*412–423.

Airaksinen, M.S., S. Alanen, E. Szabat, T.J. Visser, and P. Panula (1992) Multiple neurotransmitters in the tuberomammillary nucleus: Comparison of rat, mouse and, guinea pig. J. Comp. Neurol. *323:*103–116.

Baguna, J., and R. Ballester (1978) The nervous system in planarians: Peripheral and gastrodermal plexuses, pharynx innervation, and the relationship between central nervous system structure and the acoelomate organization. J. Morphol. *155:*237–252.

Barnard, E.A., M.G. Darlison, and P. Seeburg (1987) Molecular biology of the $GABA_A$ receptor: the receptor/channel superfamily. TINS *10:*502–509.

Barnes, R.S.K., P. Calow, and P.J.W. Olive (1988) The Invertebrates: A New Synthesis. Oxford: Blackwell Scientific.

Cooke, I.R.C., and A. Gelperin (1988) Distribution of GABA-like immunoreactive neurons in the slug *Limax maximus*. Cell Tissue Res. *253:*77–81.

Coons, A.H. (1958) Fluorescent antibody methods. In J.F. Danielli (ed): General Cytochemical Methods. New York: Academic Press, pp. 399–442.

Eriksson, B.-M. and B.-A. Persson (1982) Determination of catecholamines in rat heart tissue and plasma samples by liquid chromatography with electrochemical detection. J. Chromatogr. *228:*143–154.

Eriksson, K., M. Reuter, and O. Timoshkin (1993) Analysis of catecholamines in turbellarians of Lake Baikal. Comp. Biochem. Physiol. *105C:* 509–511.

Field, K.G., G.J. Olsen, D.J. Lane, S.J. Giovannoni, M.T. Ghiselin, E.C. Raff, N.R. Pace, and R.A. Raff (1988) Molecular phylogeny of the animal kingdom. Science *239:*748–753.

Gustafsson, M.K.S., and M. Reuter (1992) The map of neuronal signal substances in flatworms. In R.N. Singh (ed): Nervous Systems. Principles of Design and Function. New Delhi: Wiley Eastern Limited, pp. 165–188.

Hatt, H., and C. Franke (1989) Wide range transmitter sensitivities of a crustacean chloride channel. In P.A.V. Anderson (ed): Evolution of the First Nervous Systems. New York: Plenum Press, pp. 167–176.

Homberg, U., A. Bleick, and W. Rathmayer (1993) Immunocytochemistry of GABA and glutamic acid decarboxylase in the thoracic ganglion of the crab *Eriphia spinifrons*. Cell Tissue Res. *271:*279–288.

Johnson, C.D., and A.O.W. Stretton (1987) GABA-like immunoreactivity in inhibitory motor neurones of the nematode, *Ascaris*. J. Neurosci. *7*:223–235.

Kabotyanski, E.A., L.P. Nezlin, and D.A. Sakharov (1991) Serotonin neurones in the planarian pharynx. In D.A. Sakharov and W. Winlow (eds): Simpler Nervous Systems. Studies in Neuroscience 13. Manchester: Manchester University Press, pp. 138–152.

Karhunen, T., M.S. Airaksinen, L. Tuomisto, and P. Panula (1993) Neurotransmitters in the nervous system of *Macoma balthica* (Bivalvia). J. Comp. Neurol. *334*:477–488.

Keenan, L., H. Koopowitz, and K. Bernardo (1979) Primitive nervous systems: Action of aminergic drugs and blocking agents on activity in the ventral nerve cord of the flatworm *Notoplana acticola*. J. Neurobiol. *10*:397–408.

King, W., and D.O. Carpenter (1987) Distinct GABA and glutamate receptors may share a common channel in *Aplysia* neurons. Neurosci. Lett. *82*:343–348.

Maule, A.G., C. Shaw, D.W. Halton, L. Thim, C.F. Johnston, I. Fairweather, and K.D. Buchanan (1991) Neuropeptide F: A novel parasitic flatworm regulatory peptide from *Moniezia expansa* (Cestoda: Cyclophyllidea). Parasitology *102*:309–316.

Maule, A.G., C. Shaw, D.W. Halton, and L. Thim (1993) GNFFRFamide: A novel FMRFamide-immunoreactive peptide isolated from the sheep tapeworm, *Moniezia expansa*. Biochem. Biophys. Res. Commun. *193*: 1054–1060.

McIntire, S.L., E. Jorgensen, J. Kaplan, and H.R. Horvitz (1993) The GABAergic nervous system of *Caenorhabditis elegans*. Nature *364*:337–341.

Morita, M., and J.B. Best (1966) Electron microscopic studies of planaria III. Some observations on the fine structure of planarian nervous tissue. J. Exp. Zool. *161*:391–412.

Morita, M., F. Hall, J.B. Best, and W. Gern (1987) Photoperiodic modulation of cephalic melatonin in planarians. J. Exp. Zool. *241*:383–388.

Panula, P., O. Häppölä, M.S. Airaksinen, S. Auvinen, and A. Virkamäki (1988) Carbodiimide as a tissue fixative in histamine immunohistochemistry and its application to developmental neurobiology. J. Histochem. Cytochem. *36*:259–270.

Pax, R.A., and J.L. Bennett (1991) Neurobiology of parasitic platyhelminths: Possible solutions to the problems of correlating structure with function. Parasitology *102*:S31–S39.

Raether, W. (1988) Chemotherapy and other control measures of parasitic diseases in domestic animals and man. In H. Mehlhorn (ed): Parasitology in Focus: Facts and Trends. Berlin: Springer, pp. 739–866.

Roberts, E. (1986) GABA: The road to neurotransmitter status. In R.W. Olsen and J.C. Venter (eds): Benzodiazepine/GABA Receptors and Chloride Channels. Structural and Functional Properties. New York: Alan R. Liss, Inc., pp. 1–39.

Szabat, E., S. Soinila, O. Häppölä, A. Linnala, and I. Virtanen (1992) A new monoclonal antibody against the GABA-protein conjugate shows immunoreactivity in sensory neurons of the rat. Neuroscience *47*:409–420.

Tamura, H., T.P. Hicks, Y. Hata, T. Tsumoto, and A. Yamatodani (1990) Release of glutamate and aspartate from the visual cortex of the cat following activation of afferent pathways. Exp. Brain Res. *80*:447–455.

Usherwood, P.N.R., and H. Grundfest (1965) Peripheral inhibition in skeletal muscle of insects. J. Neurophysiol. *28*:497–518.

Venturini, G., F. Stocchi, V. Margotta, S. Ruggieri, D. Bravi, P. Bella, and G. Palladini (1989) A pharmacological study of dopaminergic receptors in planaria. Neuropharmacology *28*:1377–1382.

Walker, R.J., and L. Holden-Dye (1991) Evolutionary aspects of transmitter molecules, their receptors and channels. Parasitology *102*:S7–S29.

Watson, A.D.H. (1986) The distribution of GABA-like immunoreactivity in the thoracic nervous system of the locust *Schistocerca gregaria*. Cell Tissue Res. *246*:331–341.

Webb, R.A. (1988) Endocrinology of acoelomates. In H. Laufer and R.G.H. Downer (eds): Endocrinology of Selected Invertebrate Types. New York: Alan R. Liss, Inc., pp. 31–62.

Webb, R.A., and H. Eklove (1989) Demonstration of intense glutamate-like immunoreactivity in the longitudinal nerve cords of the cestode *Hymenolepis diminuta*. Parasitol. Res. *75*:545–548.

Welsh, J.H. (1972) Catecholamines in the invertebrates. In H. Blaschko and E. Muscholl (eds): Handbook of Experimental Pharmacology 33. Berlin: Springer, pp. 79–109.

Welsh, J.H., and L.D. Williams (1970) Monoamine-containing neurons in planaria. J. Comp. Neurol. *138*:103–116.