

GABA as a Neurotransmitter in Gastropod Molluscs

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Abstract. The neurotransmitter gamma-aminobutyric acid (GABA) is widely distributed in the mammalian central nervous system, where it acts as a major mediator of synaptic inhibition. GABA also serves as a neurotransmitter in a range of invertebrate phyla, including arthropods, echinoderms, annelids, nematodes, and platyhelminthes. This article reviews evidence supporting the neurotransmitter role of GABA in gastropod molluscs, with an emphasis on its presence in identified neurons and well-characterized neural circuits. The collective findings indicate that GABAergic signaling participates in the selection and specification of motor programs, as well as the bilateral coordination of motor circuits. While relatively few in number, GABAergic neurons can influence neural circuits *via* inhibitory, excitatory, and modulatory synaptic actions. GABA's colocalization with peptidergic and classical neurotransmitters can broaden its integrative capacity. The functional properties of GABAergic neurons in simpler gastropod systems may provide insight into the role of this neurotransmitter phenotype in more complex brains.

Introduction

GABA is the major inhibitory neurotransmitter in the mammalian brain (Roberts, 1960, 1986a, b; Florey, 1961; Krnjević, 1970). Perturbation of GABAergic signaling has been implicated in numerous neurological disorders, including epilepsy, Parkinson's disease, and Huntington's disease (Watts *et al.*,

2012; Möhler, 2013; Johnston *et al.*, 2016). GABA also acts as a neurotransmitter in several invertebrate phyla, including arthropods (Kuffler and Edwards, 1958; Kravitz *et al.*, 1963; Otsuka *et al.*, 1967), echinoderms (Newman and Thorndyke, 1994), annelids (Ito *et al.*, 1969; Cline, 1983, 1986), nematodes (del Castillo *et al.*, 1964; Johnson and Stretton, 1987; McIntire *et al.*, 1993), and platyhelminthes (Eriksson and Panula, 1994). Moreover, GABA has been shown to produce contractile responses in sponges (Porifera), raising the possibility that its signaling function preceded the appearance of nervous systems (Ellwanger *et al.*, 2007; Elliott and Leys, 2010; Nickel, 2010). The conserved role of GABA as a neurotransmitter across phylogeny supports its ancient origins and ubiquitous function in the core operation of neural circuits.

The nervous systems of gastropod molluscs provide experimentally favorable models for investigating the organization of action (Kandel, 1976, 1979; Davis and Gillette, 1978; Chase, 2002), neuroendocrine regulation of behavior (Kupfermann, 1970; Roubos, 1976; Conn and Kaczmarek, 1989), principles of motor control (Kupfermann and Weiss, 1978; Getting and Degin, 1985; Katz, 1995), and the cellular basis of learning and memory (Kandel, 1970, 2001; Crow and Alkon, 1980; Benjamin *et al.*, 2008). These nervous systems contain large identifiable neurons that can be classified according to neurotransmitter phenotype (Pentreath *et al.*, 1974; Ono and McCaman, 1980, 1984; Church and Lloyd, 1991). This article reviews evidence accumulated over the past 50 years that supports the role of GABA as a neurotransmitter in gastropod molluscs. GABAergic signaling by identified neurons in circuits that control behavior is emphasized because this role could provide insights into functions that are generalizable across phylogeny.

Biochemical Foundations

In his landmark review of invertebrate neurotransmitters, Gerschenfeld (1973, p. 81) concluded that the "possibility that GABA may play a role as a transmitter in the snail is very re-

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Abbreviations: BCI, buccal-cerebral interneuron; CBC, cerebral-buccal connective; CBI, cerebral-buccal interneuron; CNS, central nervous system; CPG, central pattern generator; Cr-Aint, cerebral A interneuron; DA, dopamine; EPSP, excitatory postsynaptic potential; FCAP, feeding circuit activating peptide; GABA, gamma-aminobutyric acid; GABA_{li}, GABA-like immunoreactivity; IPSP, inhibitory postsynaptic potential; PKC, protein kinase C.

mote on the basis of present knowledge." In several prior reports, GABA was shown to produce both excitatory and inhibitory responses upon application to snail neurons (Gerschenfeld and Tauc, 1961; Kerkut and Walker, 1961; Gerschenfeld and Lasansky, 1964; see Fig. 1A₁, A₂). However, Gerschenfeld's reticence to confer neurotransmitter status reflected the failure of contemporaneous biochemical investigations to detect GABA or glutamic acid decarboxylase activity in snail ganglia (Roberts, 1960; Kerkut and Cottrell, 1962; Bradford *et al.*, 1969).

Subsequent refinements to microanalytical separation and detection methods revealed low levels of GABA in individual ganglia of *Helix pomatia* (Osborne *et al.*, 1971; Dolezalova *et al.*, 1973). The presence of GABA in individual neurons was also reported in *H. pomatia* and *Aplysia* (Briell *et al.*, 1971; Osborne *et al.*, 1971; Cottrell, 1974). High-performance

liquid chromatography enabled quantification of GABA levels in individual ganglia, establishing its differential regional distribution in the central nervous system (CNS) of *Helisoma* (Richmond *et al.*, 1991). The demonstration of glutamic acid decarboxylase activity in the brain of *H. pomatia* provided a mechanism for GABA synthesis in the gastropod nervous system (Osborne *et al.*, 1971). In *Helisoma trivolvis*, synthesis of ³H-GABA from ³H-glutamate was shown to occur predominantly in the buccal, cerebral, and pedal ganglia, consistent with its immunohistochemical localization (Richmond *et al.*, 1991; see Neuronal Localization).

The capacity for GABA uptake was supported by the demonstration of a high-affinity ($K_m = 52 \mu\text{mol L}^{-1}$), sodium-dependent GABA transport mechanism in the CNS of *Aplysia dactylomela* (Zeman *et al.*, 1975). Autoradiography disclosed GABA uptake and accumulation in the central ganglia of *Aplysia* (Zeman *et al.*, 1975) and in the pond snails *Planorbis corneus* (Turner and Cottrell, 1978) and *H. trivolvis* (Richmond *et al.*, 1991). In *Aplysia*, GABA accumulation occurred predominantly in glia, suggesting a functional role for glial uptake at GABAergic synapses (Zeman *et al.*, 1975). In *Helisoma*, GABA uptake occurred in neurons that corresponded to cells labeled with GABA immunohistochemistry (Richmond *et al.*, 1991).

Thus, during the years following Gerschenfeld's 1973 review, evidence for the presence, synthesis, and uptake of GABA in gastropod nervous systems fulfilled several criteria required for its designation as a neurotransmitter. These foundations paved the way for studies aimed at characterizing GABA receptors and the localization of GABA to individual neurons.

GABA Receptors: Pharmacology and Molecular Biology

Focal delivery with iontophoretic ejection from micropipettes revealed properties of GABA receptors on gastropod neurons. Early investigations showed that GABA could produce both excitatory and inhibitory responses (Gerschenfeld and Lasansky, 1964; Walker *et al.*, 1971, 1975; Takeuchi *et al.*, 1977; Vehovsky *et al.*, 1989). Testing individual identified neurons in *Aplysia*, Yarowsky and Carpenter (1977, 1978b) established five classes of GABA responses, including (1) a rapid Cl⁻ dependent hyperpolarization, (2) a slower K⁺ dependent hyperpolarization, (3) a rapid curare sensitive depolarization, (4) a slower curare insensitive depolarization, and (5) a slow depolarization that resulted from a decreased K⁺ conductance. Several observations suggested that these receptors were utilized by synapses: (1) they were observed on only a subset of neurons; (2) characteristic response profiles were consistently observed on specific identified neurons; and (3) response types were localized to specific regions of reactive neurons. The slow K⁺ dependent responses were restricted to the neuropil, further supporting their involvement in synaptic signaling (Yarowsky and Carpenter, 1978b).

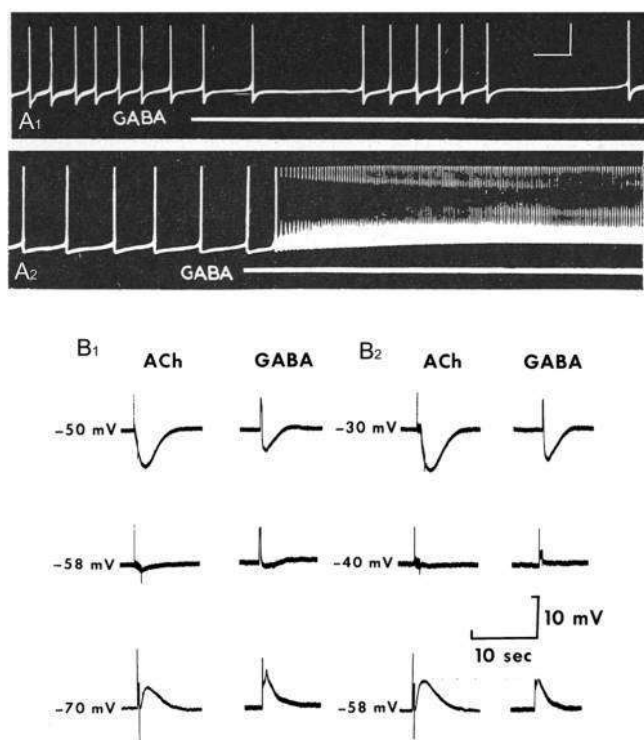


Figure 1. GABA as a neurotransmitter in gastropod molluscs. (A₁, A₂) Initial demonstration of inhibitory (A₁) and excitatory (A₂) actions of GABA on gastropod neurons. Perfusion of GABA (1 mmol L^{-1} ; white lines under recordings) while recording from neurons in the viscerio-abdominal ganglia of *Helix aspersa*. Calibration bars: 2 s, 25 mV, from another panel in original paper, apply to both (A₁) and (A₂). Reprinted from Gerschenfeld and Lasansky, 1964. *Int. J. Neuropharmacol.* **3**: 301–314, with permission from Elsevier. (B₁, B₂) Common properties of acetylcholine and GABA responses on cell R2 of *Aplysia*. (B₁) In control solution, $[\text{Cl}^-] = 593 \text{ mmol L}^{-1}$, the reversal potential for both ACh and GABA was near -58 mV . (B₂) When the external Cl⁻ concentration was reduced to 296 mmol L^{-1} , the reversal potential for both drugs was shifted to -40 mV . ACh and GABA were delivered from independent iontophoretic pipettes. Reprinted from Yarowsky and Carpenter, 1978. *J. Neurophysiol.* **41**: 531–541, with permission from the American Physiological Society.

The rapid Cl^- conductance increase elicited by GABA in the *Aplysia* visceral ganglion was found to share several characteristics with responses elicited by acetylcholine (Yarowsky and Carpenter, 1978a). Both responses reversed at -58 mV, and both exhibited similar permeability profiles (Fig. 1B₁, B₂). While both responses were blocked by the classical GABA antagonists picrotoxin and bicuculline, they did not cross-desensitize, and only the acetylcholine (ACh) response was blocked by α -bungarotoxin and strychnine. These results were consistent with the prescient hypothesis advanced by Swann and Carpenter (1975, p. 754) that “the ionophores associated with receptors to different neurotransmitters share many common properties, and may, in fact, be identical” (Yarowsky and Carpenter, 1978a; see also King and Carpenter, 1987, 1989).

The emergence of molecular biological and omics approaches confirmed that neurotransmitter receptors do, in fact, belong to large superfamilies that share common structural and functional properties (see Hille, 1989; Walker *et al.*, 1996; Changeux, 2012). Early evidence for conservation of function of ligand-gated receptors emerged from studies of the GABA_A receptor β subunit of *Lymnaea stagnalis* (Harvey *et al.*, 1989, 1991). Co-expression of the *Lymnaea* β subunit with the bovine GABA_A $\alpha 1$ subunit in *Xenopus* oocytes resulted in functional hetero-oligomeric receptors (Harvey *et al.*, 1991).

More recently, genes encoding GABA receptors and other proteins involved in GABAergic signaling were identified in genomic and transcriptomic data generated from *Aplysia californica* (Moroz *et al.*, 2006), *Lymnaea stagnalis* (Feng *et al.*, 2009; Sadamoto *et al.*, 2012), *Biomphalaria glabrata* (Adema *et al.*, 2017), and *Biomphalaria alexandrina* (Mansour *et al.*, 2017). Analysis of a *Tritonia diomedea* CNS transcriptome disclosed a β subunit of the GABA_A receptor, a GABA_{B1} metabotropic receptor, glutamate decarboxylase, a vesicular GABA transporter, and a plasma membrane GABA transporter (Senatore *et al.*, 2015). Genomic and transcriptomic approaches thus firmly established the presence of the molecular machinery for GABAergic synaptic signaling in gastropod nervous systems.

Neuronal Localization

Development of GABA-specific antibodies led to the histological localization of putative GABAergic neurons in several gastropod taxa. GABA-like immunoreactivity (GABA_{li}) was initially localized to small neurons in the buccal, cerebral, and pedal ganglia in the terrestrial slug *Limax maximus* (Cooke and Gelperin, 1988). Similar distributions of GABA_{li} neurons were observed in other panpulmonates, including the land snails *Helix pomatia* (Hernádi, 1994) and *Helix aspersa* (Jerusalimsky and Balaban, 2001) and the aquatic snails *Helisoma trivolvis* (Richmond *et al.*, 1991) and *Biomphalaria glabrata* (Vaasjo *et al.*, 2018). In *Lymnaea stagnalis*, a broader distribution of GABA_{li} neurons was reported, including cells in the right parietal and visceral ganglion (Hatakeyama and Ito, 2000).

As part of a recent examination of GABA-dopamine colocalization in pulmonates (Vaasjo *et al.*, 2018; see Colocalization of GABA and Dopamine), we confirmed the presence of a GABA-like epitope in neurons in the subesophageal ganglia of *Lymnaea* (Hatakeyama and Ito, 2000; Fig. 2A). One GABA_{li} cell, a giant neuron in the ventromedial visceral ganglion, exhibited rhythmic spiking (Fig. 2B₁). The axon of this cell bifurcated within the visceral ganglion and projected to the two pedal ganglia (Fig. 2C₁–C₃). Because of its size and position near the edge of the ganglion, this cell could usually be visualized from both ventral and dorsal aspects. We tentatively designate it VD1, according to the map of Benjamin and Winlow (1981). The large GABA_{li} cell on the posteromedial edge of the right parietal ganglion (Fig. 2A, B₂, D₁–D₃) is proposed to correspond to RPD2, a cell with electrophysiological properties and neurotransmitter contents in common with VD1 (Fig. 2B₂, D₁–D₃; Soffe and Benjamin, 1980; Wildering *et al.*, 1991; Kerkhoven *et al.*, 1992).

In each of the panpulmonate species examined, GABAergic fiber systems were largely confined to the CNS (Cooke and Gelperin, 1988; Richmond *et al.*, 1991; Hatakeyama and Ito, 2000). Some exceptions include a projection of GABA_{li} fibers to the lips of *H. pomatia* (Hernádi, 1994), a fiber system in the aorta of *H. aspersa* (Jerusalimsky and Balaban, 2001), and a projection to the base of the tentacle in *Biomphalaria* (Vaasjo *et al.*, 2018). Fibers were located in each of the interganglionic connectives, and major GABA_{li} tracts were present in the commissures connecting the paired buccal, cerebral, and pedal hemiganglia. Collectively, the patterns of GABA_{li} in panpulmonates supported a role of GABAergic signaling in the central regulation and selection of behaviors, as well as in the bilateral coordination of sensorimotor systems (Arshavsky *et al.*, 1993).

General features of the distribution of GABA_{li} in panpulmonates were also observed in other gastropod groups. GABA_{li} neurons were detected in the buccal, cerebral, and pedal ganglia of the marine euopisthobranchs *Clione limacina* (Arshavsky *et al.*, 1993) and *Aplysia californica* (Soinila and Mpitsos, 1991; Díaz-Ríos *et al.*, 1999) and in the nudibranchs *Tritonia diomedea*, *Melibe leonina*, *Dendronotus iris*, and *Hermisenda crassicornis* (Gunaratne *et al.*, 2014; Gunaratne and Katz, 2016; Webber *et al.*, 2017). A detailed comparison of GABA_{li} in the buccal ganglia of nudibranchs revealed a consistent pattern across species with widely varying feeding behaviors (Gunaratne and Katz, 2016). GABA_{li} in a sister Nudipleura species, *Pleurobranchia californica* (Pleurobranchomorpha), was highly divergent, however, leading to the proposal that it could represent a derived feature related to its specialized cannibalistic feeding behavior (Gunaratne and Katz, 2016).

GABAergic Synaptic Signaling and Regulation of Behavior

Involvement of GABA in the regulation of gastropod feeding was initially suggested by electrophysiological and behav-

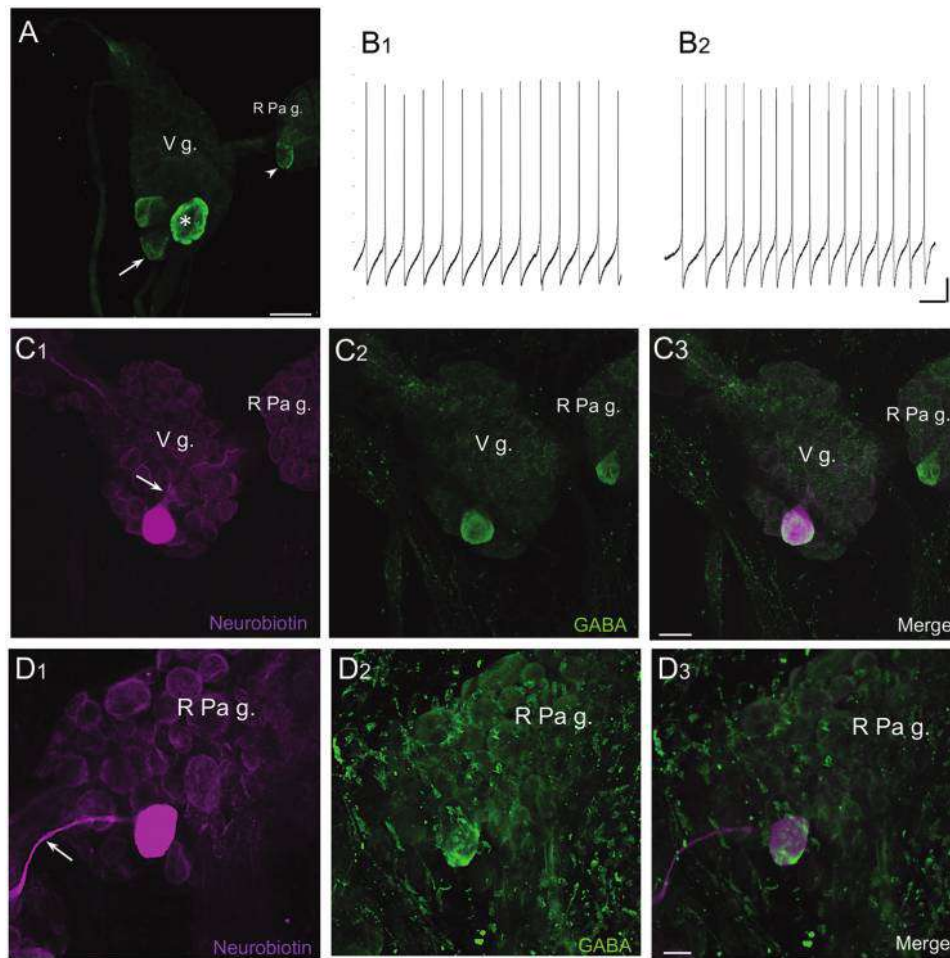


Figure 2. GABA-like immunoreactive (GABA_{li}) neurons in the subesophageal ganglia of *Lymnaea stagnalis*. (A) GABA_{li} neurons in the visceral ganglion (V g.) and right parietal ganglion (R Pa g.). A single large cell (20–30 μ m, arrowhead) was located at the posterior edge of the right parietal ganglion, and a group of three cells (arrow) was located at the posterior pole of the visceral ganglion. One of the visceral ganglion cells was significantly larger (40–50 μ m, asterisk). Calibration bar = 50 μ m. (B₁) Intracellular recording from the large putative VD1 neuron in the visceral ganglion disclosed rhythmic spiking activity. (B₂) Repetitive impulses were also recorded from cell RPD2. Calibration bars = 2 s, 10 mV. (C₁) Injection of the large visceral GABA_{li} neuron with neurobiotin showed branching of its axon (arrow) and projections toward the right and left parietal ganglia. (C₂) Same field of view as (C₁), after processing for GABA-like immunoreactivity. (C₃) Overlay of (C₁) and (C₂). Merge of magenta and green appears white. Calibration bar = 50 μ m, applies to (C₁–C₃). (D₁) Injection of RPD2 with neurobiotin labeled a projection toward the visceral ganglion (arrow). (D₂) Same field of view as (D₁) after processing for GABA-like immunoreactivity. (D₃) Merge of (D₁) and (D₂). Calibration bar = 20 μ m, applies to (D₁–D₃).

ioral studies. Application of GABA to the buccal ganglion of *Limax maximus* was reported to suppress the intensity of feeding motor programs elicited by stimulation to the lips (Cooke *et al.*, 1985). Injection of GABA into the hemocoel of *Clione limacina*, however, evoked elements of its complex predatory behavior, including tentacle protraction, mouth opening, and rhythmic movements of the buccal mass (Arshavsky *et al.*, 1991, 1993). In the isolated CNS, GABA activated (1) motor neurons in the cerebral ganglion responsible for protraction of the tentacles, or buccal cones (see also Norekian and Satterlie, 1993); (2) the feeding rhythm generator in the buccal ganglion;

and (3) efferent input to statocyst receptor cells. Excitatory actions on the buccal motor network were mimicked by baclofen and were proposed to reflect activation of GABA_B-like receptors (Arshavsky *et al.*, 1993; see also Richmond *et al.*, 1994). Collectively, GABA appeared capable of coordinating multiple motor systems required to achieve the highly complex feeding behavior of *Clione* (Arshavsky *et al.*, 1993). GABA produced similar organizational effects in the land snail *Helix lucorum*, where it promoted feeding movements and inhibited the neural circuit controlling an incompatible behavior: defensive withdrawal (Bravarenko *et al.*, 2001).

The prey capture component of *Clione* feeding provided the first demonstration of GABAergic synaptic signaling by identified gastropod neurons (Norekian, 1999; Fig. 3A₁, A₂; Table 1). A pair of GABAergic neurons (cerebral A interneuron [Cr-Aint], Arshavsky *et al.*, 1993; Norekian and Satterlie, 1993) was shown to drive a prolonged afterdischarge in the network that controls the buccal cone appendages. Each Cr-Aint projects a prominent axon through the cerebral commissure and produces excitation of its contralateral counterpart *via* electrical coupling and excitatory GABAergic synapses. Each Cr-Aint also produces prolonged self-excitatory GABAergic afterpotentials. The electrical coupling and recurrent excitation between the two Cr-Aint cells, as well as their autaptic self-excitation, were proposed to drive the long-lasting afterdischarge in the cerebral A motor neurons that project to the prey capture appendages (Norekian, 1993, 1999).

GABAergic neurons are also present in the feeding system of *Aplysia*, where they play multiple roles in the regulation of motor activity. Two commissural GABAergic interneurons in each buccal hemiganglion, termed B34 and B40, participate in shaping feeding motor programs (Hurwitz *et al.*, 1997; Jing and Weiss, 2001, 2002; Jing *et al.*, 2003; Sasaki *et al.*, 2009). Both B34 and B40 project axons across the buccal

commissure and exert their strongest synaptic actions in the contralateral hemiganglion. They also project to the cerebral ganglion *via* the contralateral cerebral-buccal connective (CBC), but their role as buccal-cerebral interneurons (BCIs) remains largely unexplored. In postsynaptic buccal cells, B34 and B40 produce chloride-mediated rapid inhibitory postsynaptic potentials (IPSPs) that are desensitized by GABA and the GABA_A agonist muscimol, blocked by the GABA_A antagonists picrotoxin and bicuculline, and augmented by the GABA uptake inhibitor nipecotic acid (Jing *et al.*, 2003).

Rapid IPSPs produced by B34 and B40 influence the multifunctional two-phase (radula protraction/retraction) motor program toward its ingestive conformation by prolonging the protraction phase of the program (Jing *et al.*, 2003). Rapid GABAergic IPSPs elicited by B40 in interneurons and motor neurons also bias motor programs toward ingestion by coordinating closure of the radula with its retraction phase, resulting in an inward displacement of food toward the esophagus (Jing *et al.*, 2003). Slower and more long-lasting excitatory signals from B40 are mediated *via* GABA_B-like receptors on radula closer motor neurons (Dacks and Weiss, 2013). Because these synaptic responses also bias the circuit toward its ingestive conformation, it was concluded that inhibitory and excitatory

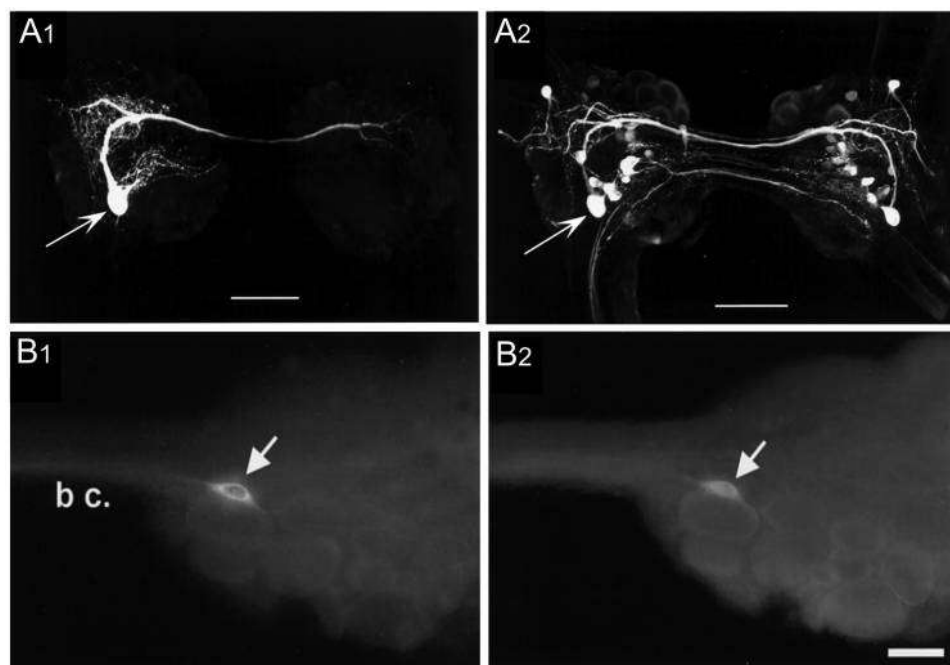


Figure 3. Identified GABAergic neurons. (A₁, A₂) Identified GABAergic neuron cerebral A interneuron (Cr-Aint) in the prey capture circuit of *Clione limacia*. (A₁) A single CR-Aint (arrow) was filled with neurobiotin and visualized with Texas Red-labeled avidin. (A₂) GABA-like immunoreactivity, same cerebral ganglion as (A₁). The cell body of Cr-Aint is indicated by the arrow. Scale bars = 200 μ m. Reprinted from Norekian, 1999. *J. Neurosci.* **19**: 1863–1875, with permission from the Society for Neuroscience. (B₁, B₂) Colocalization of THli and GABAergic neurons in neuron B20 in the buccal ganglion of *Aplysia californica*. (B₁) THli was observed in one neuron (arrow) on the rostral surface of each buccal hemiganglion (only left hemiganglion shown) near the buccal commissure (b.c.). (B₂) GABAergic neurons were present in the same cell (arrow). Calibration bar = 40 μ m, applies to both (B₁) and (B₂). Reprinted from Díaz-Ríos and Miller, 2002. *J. Comp. Neurol.* **445**: 29–46, with permission from John Wiley.

Table 1

Identified GABAergic neurons in gastropods

Species	Neuron	Cotransmitter	Commissural	BCI	CBI	Reference
<i>Clione limacina</i>	Cr-Aint		✓			Norekian, 1999
	Cr-BM				✓	Norekian and Malyshev, 2005
<i>Aplysia californica</i>	B34	ACh ^a	✓	✓		Jing and Weiss, 2003
	B40		✓	✓		Jing and Weiss, 2003
	B20	Dopamine	✓	✓		Díaz-Ríos <i>et al.</i> , 2002
	B65	Dopamine	✓			Díaz-Ríos <i>et al.</i> , 2002
	CBI-3	APGWa			✓	Jing and Weiss, 2003
	CBI-11	FCAP, ACh ^a			✓	Wu <i>et al.</i> , 2003, 2014
<i>Aplysia kurodai</i>	CBM3				✓	Narusuye <i>et al.</i> , 2005
<i>Helisoma trivolvis</i>	N1a ^a	Dopamine	✓			Vaasjo <i>et al.</i> , 2018
	N1b ^a	Dopamine	✓	✓		Vaasjo <i>et al.</i> , 2018
<i>Lymnaea stagnalis</i>	VD1 ^a	VD1/RPD2 peptides				This article
	RPD2 ^a	VD1/RPD2 peptides				This article

BCI, buccal-cerebral interneuron; CBI, cerebral-buccal interneuron.

^a Requires confirmation.

GABAergic signals originating from a single interneuron can act over multiple timescales to influence motor circuit output in a coherent fashion (Dacks and Weiss, 2013).

In addition to its intraganglionic role in bilateral coordination of feeding in *Aplysia*, GABAergic signaling also contributes to motor program generation and specification via interganglionic projections from the cerebral ganglion. In *Aplysia californica*, approximately 13 bilaterally paired cerebral-buccal interneurons (CBIs) project to the buccal ganglion via each CBC (Rosen *et al.*, 1991). Two CBIs, termed CBI-3 and CBI-11, exhibit GABA-like immunoreactivity (Jing *et al.*, 2003; Wu *et al.*, 2003). While CBI-3 and CBI-11 are not highly effective initiators of buccal motor programs, they both can influence these programs toward their ingestive mode (Jing *et al.*, 2003; Wu *et al.*, 2003, 2014).

CBI-3 biases feeding motor patterns toward an ingestive configuration through signaling by at least two colocalized neurotransmitters, the neuropeptide APGWamide and GABA (Morgan *et al.*, 2002; Jing *et al.*, 2003). Rapid IPSPs produced by CBI-3 in the ingestive buccal interneuron B20 (see Colocalization of GABA and Dopamine) were blocked by picrotoxin and bicuculline (Jing *et al.*, 2003). CBI-3 also produces rapid excitatory postsynaptic potentials (EPSPs) in B40, resulting in feedforward inhibition of ingestive motor patterns. It was hypothesized that the rapid IPSPs and EPSPs produced by CBI-3 in the buccal ganglion are both mediated by GABA (Jing and Weiss, 2003). Further characterization of the CBI-3-elicited EPSPs could test this proposal.

A GABAergic CBI, designated CBM3, was identified in *Aplysia kurodai* and was proposed to correspond to CBI-3 of

A. californica (Narusuye *et al.*, 2005). Calcium imaging demonstrated longer-lasting responses in CBM3 when the lips were exposed to a palatable seaweed versus an aversive seaweed. This differential response to sensory stimuli supports the role of GABAergic CBIs in biasing motor programs toward their ingestive conformation (Narusuye *et al.*, 2005).

Stimulation of GABAergic CBI-11 can also bias motor programs toward their ingestive configuration in *A. californica* (Wu *et al.*, 2003, 2014). Fast picrotoxin-sensitive IPSPs in buccal motor neuron B3 produced by CBI-11 were mimicked by local application of GABA (Wu *et al.*, 2003). GABA produced a picrotoxin-sensitive hyperpolarization of B3 that reversed at the same membrane potential as the IPSPs elicited by stimulation of CBI-11 (Wu *et al.*, 2003). Further studies have emphasized the capacity of CBI-11 to specify buccal motor programs elicited by other CBI program initiators (Wu *et al.*, 2014). Its ability to bias motor programs toward the ingestive conformation was attributed to its co-expression of the feeding circuit activating peptide (FCAP) family of neuropeptides (Wu *et al.*, 2014). As described above for CBI-3, CBI-11 also produced rapid EPSPs in the ingestive GABAergic buccal interneuron B40. These EPSPs were blocked by hexamethonium, leading to the proposal that CBI-11 signaling could be mediated by acetylcholine, in addition to GABA and FCAP (Wu *et al.*, 2014; see Table 1).

A GABAergic CBI identified in *Clione limacina*, termed Cr-BM, shares several characteristics with CBI-11 of *A. californica* (Norekian and Malyshev, 2005). Cr-BM promotes ingestive motor programs by coordinating movements of three structures: the buccal cones used for prey capture, the chitin-

ous hooks used to extract the prey from its shell, and the radula. Depolarizing and hyperpolarizing postsynaptic potentials from Cr-BM to buccal motor neurons and interneurons were occluded by GABA and blocked by picrotoxin and bicuculline. It was proposed that Cr-BM is homologous to CBI-11 of *Aplysia* but that it has assumed control of additional circuits that are unique to the carnivorous feeding behavior of *Clione* (Norekian and Malyshev, 2005).

Colocalization of GABA and Dopamine

GABA-like immunoreactivity was reported to be colocalized with tyrosine hydroxylase-like immunoreactivity (THli) in five neurons in the buccal ganglion of *Aplysia* (Díaz-Ríos *et al.*, 2002). Two of the GABA_{li}-THli neurons corresponded to the previously characterized bilateral pair of dopaminergic B20 BCIs (Teyke *et al.*, 1993; Fig. 3B₁, B₂). Like the GABAergic B40 and B34 interneurons previously described, B20 fires during the initial protraction phase of buccal motor programs. In contrast to B40 and B34, however, B20 produces EPSPs, rather than IPSPs, in the radula closer motor neuron B8. By promoting radula closure during the protraction phase, B20 biases motor programs toward their egestive conformation, pushing inedible material in the outward direction (Jing and Weiss, 2001; Proekt *et al.*, 2004). The EPSPs produced by B20 in B8 were blocked by sulpiride and were occluded by dopamine, indicating that they are mediated by dopamine (Díaz-Ríos and Miller, 2005). These EPSPs were augmented by GABA and baclofen, suggesting that co-released GABA could act as a modulator by activating GABA_B receptors (Díaz-Ríos and Miller, 2005; Fig. 4A₁, A₂). Pharmacological experiments provided further support for this modulatory role of co-released GABA at the B20-to-B8 synapse, and they implicated a postsynaptic site for this action (Svensson *et al.*, 2014). Potentiation of dopamine-induced currents in B8 by GABA and baclofen was blocked by the GABA_B antagonist phaclofen, the non-specific G-protein inhibitor GDP β S, and the protein kinase C (PKC) inhibitor chelerythrine (Fig. 4B₁, B₂). Together, these observations support the proposal that GABAergic potentiation of B20-to-B8 synaptic signals reflects a postsynaptic G-protein-mediated activation of PKC in B8 (Svensson *et al.*, 2014).

GABAergic modulation of dopaminergic B20-to-B8 signaling was further examined with repetitive stimuli that mimicked the natural form of bursting in motor programs (Díaz-Ríos and Miller, 2006). GABA and baclofen enhanced two forms of intrinsic synaptic plasticity—facilitation and summation—exhibited by the EPSPs evoked in B8 by trains of impulses in B20. These observations led to the proposal that co-released GABA could influence feeding motor programs via “homosynaptic modulatory metaplasticity” (Díaz-Ríos and Miller, 2006, p. 223).

A second pair of GABA_{li}-THli buccal neurons was identified as interneuron B65 (Díaz-Ríos *et al.*, 2002). B65 was

characterized previously as a bilaterally paired catecholaminergic neuron that projects an axon across the buccal commissure and exerts its strongest synaptic actions in the contralateral hemiganglion (Kabotyanski *et al.*, 1998). Unlike B34, B40, and B20, B65 does not project to the CBC. Firing B65 is sufficient to evoke fully coordinated motor programs in which it fires during the protraction phase (Kabotyanski *et al.*, 1998). EPSPs produced by B65 in protraction phase interneurons were occluded by dopamine and blocked by sulpiride, indicating that dopamine also acts as the mediator of synaptic signals by this dual-transmitter interneuron (Due *et al.*, 2004). GABA did not mimic or occlude synaptic potentials produced by B65 (Due *et al.*, 2004). GABA and baclofen did reduce the amplitude of EPSPs generated by B65 in the retraction phase interneuron B4/B5, again suggesting its modulatory role in neurons in which it is colocalized with dopamine (Díaz-Ríos and Miller, 2005).

Recently, GABA_{li}-THli colocalization was observed in five neurons in the buccal ganglia of three pulmonate snails: *Biomphalaria glabrata*, *Helisoma trivolvis*, and *Lymnaea stagnalis* (Vaasjo *et al.*, 2018). As in *Aplysia*, one unpaired GABA_{li}-THli cell was present near the buccal commissure. Interestingly, the unpaired GABA_{li}-THli cell was located in the right buccal hemiganglion in the sinistral snails *Biomphalaria* and *Helisoma* and in the left buccal ganglion in the dextral *Lymnaea* (Vaasjo *et al.*, 2018).

The presence of GABA_{li}-THli neurons in the buccal ganglia of pulmonates indicates that colocalization of the classical neurotransmitters GABA and dopamine in feeding central pattern generator (CPG) interneurons preceded the divergence of euopisthobranch and panpulmonate taxa. It also supports the hypothesis that heterogastropod feeding CPG networks exhibit a common universal plan (Murphy, 2001; Wentzell *et al.*, 2009). Two *Helisoma* catecholaminergic protraction phase interneurons, termed N1a and N1b, were proposed to be homologous to the B65 and B20 interneurons of *Aplysia* (Murphy, 2001). These homologies (N1a : B65 and N1b : B20) were based on cell location, morphology, synaptic connections, CPG function, and dopaminergic phenotype. While the localization of GABA_{li}-THli neurons in the *Helisoma* buccal ganglion appears to add the GABA-dopamine phenotype to the shared properties of these cells, electrophysiological and dye fill confirmation will be required for unequivocal verification (Table 1).

Conclusions and Future Directions

This overview spans more than 50 years of investigation leading to our present understanding of GABA as a neurotransmitter in gastropods. The protracted advance of this inquiry may be contrasted with the earlier characterization of GABAergic neurotransmission in crustaceans and insects, where GABA serves a major role in neuromuscular signaling (Usherwood and Grundfest, 1964; Otsuka *et al.*, 1967;

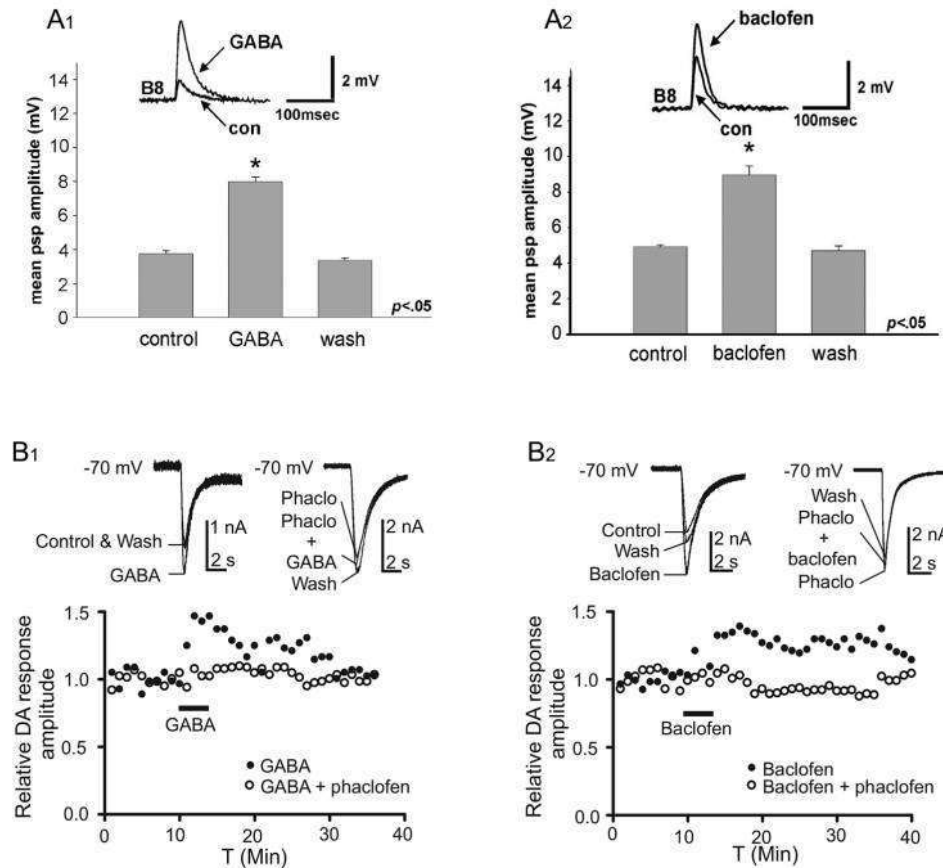


Figure 4. GABA modulates rapid dopaminergic signaling from B20 to the radula closer motor neuron B8. (A₁) Bath application of GABA (1 mmol L^{-1}) increased the amplitude of excitatory postsynaptic potentials (EPSPs) produced in B8 by impulses evoked in B20 (con, control). (A₂) GABA_B agonist baclofen (1 mmol L^{-1}) also augmented the B20-to-B8 EPSP. Reprinted from Díaz-Ríos and Miller, 2005. *J. Neurophysiol.* **93**: 2142–2156, with permission from the American Physiological Society. (B₁, B₂) GABA and baclofen potentiate dopamine (DA) currents in B8. (B₁) Perfusion of GABA ($100 \mu\text{mol L}^{-1}$) augmented the inward current evoked by dopamine puffed from a micropipette onto the soma of B8. This potentiation was blocked by the GABA_B antagonist phaclofen (Phaclo, $100 \mu\text{mol L}^{-1}$). (B₂) Perfusion of baclofen ($100 \mu\text{mol L}^{-1}$) also potentiated the inward current evoked by dopamine in B8. The effect of baclofen was also blocked by phaclofen. All experiments were conducted in the presence of tetrodotoxin ($10 \mu\text{mol L}^{-1}$) to suppress impulses and synaptic activity. Reprinted from Svensson *et al.*, 2014. *J. Neurophysiol.* **112**: 22–29, with permission from the American Physiological Society.

Sattelle, 1990; see Edwards *et al.*, 1999). In the nematode *Caenorhabditis elegans*, where GABA also functions as a neuromuscular transmitter, genetic analyses have produced a deep understanding of inhibitory and excitatory GABAergic signaling (Jorgensen, 2005). Several properties of the GABAergic phenotype of gastropods, however, including its presence in influential interneurons that are embedded in premotor circuits, are shared by the GABAergic nervous systems of vertebrates. Emerging findings are also disclosing diverse cotransmitter profiles for GABAergic neurons in the brains of mammals, including GABA's colocalization with dopamine in neurons of the olfactory bulb, retina, ventral tegmental area, and substantia nigra (Maher and Westbrook, 2008; Hirasawa *et al.*, 2012; Tritsch *et al.*, 2012, 2014, 2016). The identified GABAergic and GABA-DA interneurons of gas-

tropods should inform further investigation of these neuronal phenotypes in more complex brains.

This survey underscores the versatility of GABA as a neurotransmitter in gastropod molluscs. Although it is present in a limited number of neurons, GABAergic signaling can decisively influence circuits *via* inhibitory and excitatory synaptic actions that span a broad temporal range. The diversity of GABAergic signaling observed in gastropods may be compared with other taxa. While the GABA_A receptors of mammals are selectively permeable to anions (Enna and Bowery, 2013), the rapid excitatory GABAergic postsynaptic potentials observed in gastropods appear to reflect the presence of cation-permeable channels, as observed in nematodes (Beg and Jorgensen, 2003; Jorgensen, 2005). GABA_B receptors, which are also predominantly inhibitory in vertebrate nervous

systems (Pinard *et al.*, 2010; Enna and Bowery, 2013), are proposed to underlie excitatory modulation in the feeding networks of *Clione* (Arshavsky *et al.*, 1993), *Helisoma* (Richmond *et al.*, 1994), and *Aplysia* (Díaz-Ríos and Miller, 2005; Svensson *et al.*, 2014). Interestingly, GABA_B receptors mediate an increase of excitatory transmitter release at a crustacean neuromuscular junction (Gutovitz *et al.*, 2001), suggesting that excitatory GABA_B modulation may reflect both pre- and post-synaptic actions in invertebrates (see also Swensen *et al.*, 2000). Finally, the increasing recognition of GABA as a cotransmitter in the mammalian CNS should also stimulate investigation into the generality of GABA_B-mediated homosynaptic modulatory metaplasticity (Díaz-Ríos and Miller, 2006).

Although GABA is not present in gastropod motor neurons, it can specify both qualitative and quantitative features of motor programs (Richmond *et al.*, 1994; Moccia *et al.*, 2009; Dacks and Weiss, 2013). Likewise, despite its limited role as a sensory neurotransmitter, GABA can regulate transmission of sensory information (see Alkon *et al.*, 1993; Arshavsky *et al.*, 1993; Jin *et al.*, 2009). The versatility of GABAergic signaling in gastropods is further demonstrated by its association with diverse cotransmitters. In the *Aplysia* feeding system, it can partner with neuropeptides, for example, APGWamide in CBI-3 and FCAP in CBI-11, or with other small molecule neurotransmitters, for example, dopamine in B20 and B65 and acetylcholine in CBI-11 and B34 (Hurwitz *et al.*, 2003; see Table 1). Further study is likely to disclose additional cotransmitters in GABAergic neurons (see Díaz-Ríos and Miller, 2005; Wu *et al.*, 2014).

The localization of GABA-like immunoreactive neurons enabled early investigators to deduce several attributes of GABA's function (Cooke and Gelperin, 1988; Richmond *et al.*, 1991; Arshavsky *et al.*, 1993). GABA's presence in the paired buccal, cerebral, and pedal ganglia suggested its involvement in coordinating bilateral networks. The subsequent identification of GABAergic commissural neurons with predominant contralateral synaptic actions substantiated this proposal (Norekian, 1999; Jing and Weiss, 2003; Díaz-Ríos and Miller, 2005). The presence of GABA_A fibers in the connectives between ganglia also suggested its involvement in the control and integration of motor systems, another premise that was confirmed by behavioral studies (Arshavsky *et al.*, 1993; Bravarenko *et al.*, 2001) and with the identification of GABAergic CBIs and BCIs in the *Aplysia* and *Clione* feeding systems (Jing *et al.*, 2003; Wu *et al.*, 2003; Norekian and Malyshev, 2005).

The present state of knowledge concerning GABA as a neurotransmitter in gastropods suggests several functional properties that may be relevant to more complex nervous systems. First, its participation in bilateral motor systems ensures that the two sides of the organism perform actions in synchrony. In vertebrates, such coordination may be relevant to certain motor systems, such as breathing and chewing, where the two sides act in unison, and not to others, such as walking

and swimming, where movements on the two sides alternate. Second, the GABAergic systems of gastropods participate in circuits that can transform temporal input-out relations. Whether through bilateral reverberating excitatory afterdischarges, as in the prey capture of *Clione*, or triggering the multi-phasic feeding motor programs of *Aplysia*, GABAergic signaling can generate prolonged responses to brief stimuli. Finally, the anatomical features of the gastropod GABAergic systems suggest a role in efference copy or read-out of motor activity to higher centers. Bilateral coordination, temporal transformation, and efference copy are tasks of information processing that must be met by all complex nervous systems. The participation of GABAergic signaling in meeting these demands in the simpler gastropod nervous systems should guide investigation of this major neurotransmitter system in the vertebrate brain. This knowledge could also increase our understanding of the pathologies that can occur when this system is compromised.

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