Chapter 13
Colocalization and Cotransmission of Classical Neurotransmitters: An Invertebrate Perspective

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Abstract Once considered a curiosity, the notion that individual neurons can contain more than one classical neurotransmitter has gained increasing credibility in recent years. Several contributions to the growing recognition of classical neurotransmitter colocalization and cotransmission originate from studies using invertebrate nervous systems. Some of these model systems contain large identified neurons that contribute to well-understood circuits and networks. They therefore enable investigators to pose questions that are presently beyond the technical limitations of experimental approaches to mammalian brain function. This chapter reviews our current understanding of classical neurotransmitter colocalization and cotransmission in invertebrates. It focuses on identified neurons that could enable assessment of cotransmitter contributions to synaptic signals and neural network function. Major gaps in our present conception of classical neurotransmitter colocalization and cotransmission are emphasized, with an aim toward stimulating further study of their physiological and functional consequences.

13.1 Introduction

> *"It looks as though Mother Nature just threw a handful of neurotransmitters at the nervous system and worked with them wherever they landed."*
>
> I. Kupfermann, personal communication

In his comprehensive review of cotransmission, Kupfermann (1991) attempted to discern general principles governing combinations of classic, or conventional neurotransmitters, with neuropeptides and other substances that he classified as "unusual neurotransmitters". He concluded that "it has

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R. Gutierrez (ed.), *Co-Existence and Co-Release of Classical Neurotransmitters*,
DOI 10.1007/978-0-387-09622-3_13,

not proven possible to derive any simple rules that describe the observed combinations of cotransmitters". In the years since that article appeared, instantiations of neurotransmitter colocalization and cotransmission have increased considerably. As reviewed in this volume, these observations include instances in which classical or conventional neurotransmitters have been reported to be colocalized. Despite the increased support for its occurrence, however, many fundamental questions concerning neurotransmitter colocalization and cotransmission remain unanswered. This contribution reviews advances from studies using invertebrate nervous systems that hold promise for addressing some of the outstanding questions concerning colocalization and cotransmission of classical neurotransmitters. Evidence of colocalization in neurons that participate in well-characterized circuits is emphasized, as these models can provide opportunities to determine cotransmitter contributions to synaptic signaling, circuit function, and ultimately to behavior.

Historically, our understanding of neurotransmitters and synaptic mechanisms has benefited greatly from investigations using the large neurons found in many invertebrates (reviewed in Gerschenfeld 1973; Kandel 1975; Kuffler et al. 1984). Moreover, numerous insights into cotransmission have emerged from studies on invertebrates (Adams and O'Shea 1983; Marder et al. 1995; Nusbaum et al. 2001). Readers who are not steeped in this literature will recognize all of the classical neurotransmitters considered below as major mediators of synaptic signals in the mammalian CNS. Moreover, the biosynthetic pathways, mechanisms of release, and signal transduction pathways are, for the most part, not esoteric or idiosyncratic to the invertebrates. In view of these precedents and commonalities, it may be anticipated that findings obtained with the invertebrates can inform and facilitate efforts to decipher the functions of classical neurotransmitter colocalization and cotransmission in the mammalian brain.

Some invertebrate neurons can be recognized in all members of a particular species based upon their position, size, branching pattern, synaptic connections, firing patterns, intrinsic membrane properties, and neurotransmitter phenotype. In some instances, corresponding neurons may be found in other related species (Kandel 1979; Croll 1987). Studies of neuronal circuits composed of such "identified neurons" have advanced our understanding of numerous nervous system operations, including sensorimotor integration, central pattern generation, and plasticity (Getting 1989; Pearson 1993; Marder and Calabrese 1996). This chapter will thus emphasize the evidence for classical neurotransmitter colocalization and cotransmission in identified invertebrate neurons that contribute to neural circuit function. This focus reflects the conviction that the principles governing the operation of such "simpler" neural networks are pertinent to more complex systems.

13.2 "Giant" Serotonergic Cells

Some of the earliest studies suggesting colocalization of classical neurotransmitters in any nervous system were conducted using exceptionally large serotonergic neurons that are found in mollusks (see Burnstock 1976; Kupfermann 1991). Although distinct nomenclatures signify these neurons in different species [*Lymnaea stagnalis*: Cerebral Giant Cell (CGC); *Aplysia californica*: Metacerebral Cell (MCC); *Planorbis corneus*: Giant Serotonergic Cell (GSC), *Helix aspersa*: Giant Cerebral Neuron (GCN)] they all exhibit common structural and functional properties (see Weiss and Kupfermann 1976; Kandel 1979; Croll 1987). In all instances, their cell bodies are among the largest in the CNS. They are located in the cerebral ganglion and they project to the circuits that generate feeding behaviors. Although their serotonergic phenotype is a defining feature in all species, some observations suggesting classical cotransmitter colocalization have been reported in specific cases.

Using radioenzymatic micromethods on individual neurons dissected from *Aplysia*, Brownstein et al. (1974) measured synthesis of both serotonin and histamine in neuron C-1 (later designated the MCC). These investigators noted that their values for histamine concentrations were two orders of magnitude lower than their serotonin measurements. Subsequent studies showed that such measurements could be contaminated by the presence of presynaptic fibers and terminals that invaginate the somata of *Aplysia* neurons (Ono and McCaman 1984; see also Osborne 1979; 1984). Moreover, immunohistochemical studies did not detect histamine in the MCC of *Aplysia* (Elste et al. 1990). The MCC was found to receive innervation from a cerebral neuron that was known to be histaminergic (C2; Ono and McCaman 1980), increasing the likelihood that the Brownstein et al. (1974) originated from presynaptic sources.

Using immunohistochemistry on adjacent sections of the CGC of *Lymnaea*, Boer et al. (1984) reported colocalization of serotonin and dopamine-like immunoreactivities. In a subsequent study that mapped dopamine immunoreactivity and glyoxylic acid fluorescence in the *Lymnaea* CNS, Elekes et al. (1991) did not find evidence for the presence of DA in the CGCs. Notably, different antibodies against dopamine were used in these two investigations. Additional studies that examined the distribution of catecholamines in *Lymnaea* and additional mollusks likewise failed to detect them in the CGC or its homologs (Salimova et al. 1987; Hernádi et al. 1993; Hernádi and Elekes 1995; Sakharov et al. 1996; Croll 2001).

Thus, some of the earliest assertions of classical neurotransmitter colocalization in the giant serotonergic neurons were subsequently refuted. They are included in this survey primarily due to their impact on later studies. These investigations demonstrated how classical neurotransmitter colocalization could be erroneously inferred when based upon data obtained with a single method of detection. Consequently, they served to increase the stringency of criteria applicable to demonstrations of colocalization and cotransmission in invertebrate neurons.

13.3 Cholinergic/Serotonergic Mechanosensory Neurons

A comprehensive series of studies conducted by Katz, Harris-Warrick and coworkers provided evidence for colocalization and cotransmission of acetylcholine and serotonin in specific sensory neurons of crabs (Katz et al. 1989; Katz and Harris-Warrick 1989, 1990a, 1991; Kiehn and Harris-Warrick 1992). The gastropyloric receptor (GPR) cells, a set of four peripheral mechanosensory neurons (bilaterally paired GPR1 and GPR2 cells) in the stomatogastric nervous system of *Cancer borealis* (Jonah crab) and *Cancer irroratus* (rock crab), are activated by tension at the gastropyloric border of the foregut. They project to the stomatogastric ganglion (STG), an intensively studied central pattern generator (CPG) neuronal circuit that controls foregut movements. Measurements of choline acetyltransferase in the nerve containing the axon of GPR2 (the gastropyloric nerve, *gpn*) *en route* to its peripheral innervation were significantly above background measurements (obtained from another nerve that is not thought to contain cholinergic fibers; Katz et al. 1989). Moreover, pharmacological experiments showed that the rapid excitatory postsynaptic potentials (EPSPs) evoked by the GPRs in specific STG neurons were blocked by several nicotinic antagonists, including *d*-tubocurarine, decamethonium, hexamethonium, and mecamylamine (Katz and Harris-Warrick 1989, 1990). These data led to the proposal that ACh serves as a GPR neurotransmitter (Katz et al. 1989; Katz and Harris-Warrick 1989). In this respect, the GPRs resemble prototypical crustacean mechanosensory neurons, which use ACh as their neurotransmitter to evoke rapid EPSPs via nicotinic-like receptors in target CNS neurons (Barker et al. 1972; Hildebrand et al. 1974; Miller et al. 1992).

Immunohistochemical observations indicated that the GPRs of *Cancer* also contained serotonin and that they provided the sole source of serotonergic innervation to the STG (Katz et al. 1989; see also Beltz et al. 1984). Moreover, in addition to their rapid cholinergic signaling, the GPRs were found to exert slow modulatory actions within the STG. (Katz and Harris-Warrick 1989, 1990a; Kiehn and Harris-Warrick 1992). These modulatory GPR effects varied among the different STG neurons, with some targets responding with tonic inhibition and others responding with tonic excitation, rhythmic bursting, or plateau potentials. Each of these modulatory effects was also evoked by exogenous 5-HT introduced via bath application (Katz and Harris-Warrick 1989, 1990a) or by puffing directly to the cells (Zhang and Harris-Warrick 1994). Finally, serotonergic agonists were shown to evoke each effect and specific serotonergic antagonists were shown to block them. Each effect exhibited distinct pharmacological profiles and in all cases the ability of antagonists to block the actions of exogenous serotonin was in agreement with their ability to block the modulatory responses produced by stimulating the GPRs. These pharmacological data provided strong support for a modulatory role of serotonin in signaling by the GPRs.

In sum, these studies provided biochemical, anatomical, and pharmacological evidence for colocalization and cotransmission of acetylcholine and serotonin in the GPR neurons. The known mechanosensory function of these neurons, and their characterized projections to specific identified neurons within the STG, enabled these investigators to dissect their signaling into a rapid cholinergic component, and a slow serotonergic component, with variable effects that differed according to the receptor/transduction mechanism activated in each target.

13.4 Dopaminergic/Serotonergic Neurosecretory Cells

A complex neuron, termed the "L-cell" (Selverston et al. 1976), has been described in several crustacean species, including the crabs *Carcinus maenas* (Cooke and Goldstone 1970) and *Callinectes sapidus* (Wood and Derby 1996; Fort et al. 2004), the lobsters *Panulirus interruptus* (Kushner and Maynard 1977), *Homarus gammarus* (Cournil et al. 1984, 1994), and *Homarus americanus* (Siwicki et al. 1987; Pulver et al. 2003) and the crayfish *Oronectes rusticus* (Tierney et al. 2003). One L-cell is located in each commissural ganglion, a small aggregation of neurons located on the connective that joins the brain to the remainder of the CNS. In those species in which its anatomy has been described, the L-cell projects to the brain, where small collaterals innervate the tritocerebral neuropil. The main axon of the L-cell then reverses its course and projects in the posterior direction past its ganglion of origin, to the thoracic nervous system. Upon reaching the thoracic ganglia, the L-cell axon (termed the A fiber by Maynard, 1961) turns sharply to exit the CNS via the first segmental nerve (SN1; Cooke and Goldstone 1970; Fort et al. 2004). It projects via SN1 to the pericardial organs (POs), major neurosecretory structures that flank the heart, where it ramifies into many smaller fibers with varicose terminals positioned to release its products into the pericardial sinus. In *Callinectes sapidus*, it was proposed that a branch of the L-cell leaves the POs and projects to the heart (Fort et al. 2004), where it terminates within the cardiac ganglion (CG), a small (9 neurons) aggregate of cells that produces the neurogenic crustacean heartbeat. The extensive projections of the L-cell thus enable it to influence (1) circuits within the brain, (2) the heartbeat via its projection to the CG, and (3) systems and tissues throughout the organism that are responsive to circulating neurohormones.

The physiological properties of the L-cell were examined in the lobster *Homarus gammarus* (Robertson and Moulins 1981). It was found that the L-cell firing pattern reflected the influence of four distinct foregut rhythms and it was postulated that it provided a corollary discharge reflecting this motor output. The L-cell was also proposed to act in a feedback capacity, modifying the stomatogastric nervous system that controls the foregut via neurohormonal release from its terminals in the PO (Robertson and Moulins

1981). Synaptic actions of the L-cell have not been studied. In contrast to its neurosecretory role which has been known for some time, its projections to sites where it may exert more direct synaptic actions, e.g., the tritocerebrum (Tierney et al. 2003) and the cardiac ganglion (Fort et al. 2004) have only been recently disclosed.

A defining feature of the L-cell in all species examined to date is its catecholaminergic phenotype. Originally demonstrated using histofluorescent methods in *Carcinus maenas* (Cooke and Goldstone 1970) and *Panulirus interruptus* (Kushner and Maynard 1977), the presence of catecholamines in the L-cell was subsequently shown using antibodies to dopamine in *Homarus gammarus* (Cournil et al. 1984) and *Callinectes sapidus* (Wood and Derby 1996). Antibodies to tyrosine hydroxylase (TH), the rate-limiting enzyme in the catecholamine biosynthetic pathway have been shown to label the L-cell in *Homarus gammarus* (Cournil et al. 1984, 1994), *Callinectes sapidus* (Wood and Derby 1996; Fort et al. 2004) and *Homarus americanus* (Pulver et al. 2003).

Biochemical approaches also support the presence of catecholamines in the L-cell. Initially, radioenzymatic assays showed that the L-cell of *Panulirus interruptus* contained and accumulated dopamine (Kushner and Barker 1983). High performance liquid chromatography (HPLC) with electrochemical detection also showed high levels of DA in extracts of L-cell somata isolated from *Homarus gammarus* (Cournil et al. 1984). Importantly, the biochemical methods did not detect significant quantities of norepinephrine, suggesting that dopamine is the primary, and possibly the only, catecholamine neurotransmitter in crustaceans (Sullivan et al. 1977; Barker et al. 1979; Cooke and Sullivan 1982). Together, the accumulated evidence supports the conclusion that dopamine is present in the L-cells of all crustacean species that have thus far been examined.

In addition to dopamine, the L-cells of various decapod species also contain cotransmitters. In contrast to the apparent ubiquity of DA, however, the L-cell cotransmitter complement exhibits substantial species variability. In the lobster *Homarus americanu*s (Siwicki et al. 1987) and the crabs *Cancer irroratus* and *Cancer borealis* (Marder et al. 1986) it contains the pentapeptide proctolin. In *Homarus gammarus* (Cournil et al. 1984), several crayfish species (Tierney et al. 1999), and the prawn *Macrobrachium rosenbergii* (Sosa et al. 2002), the following observations indicate that serotonin serves as an L-cell cotransmitter: (1) Immunohistochemical experiments by Cournil et al. (1984) on serial sections of the *Homarus gammarus* commissural ganglion showed colocalization of dopamine and serotonin in the L-cell. These investigators also found that levels (4×10^{-4} M) of serotonin in isolated L-cell somata measured by radioimmunoassay were comparable to dopamine concentrations (2×10^{-4} M) measured using HPLC. Finally, it was reported that L-cells identified using electrophysiological criteria exhibited serotonin immunoreactivity (Cournil et al. 1984). (2) Tierney et al. (1999) identified a large serotonin-immunoreactive neuron in the commissural ganglia of seven crayfish species. They proposed that this neuron corresponds to the L-cell and, in at least one case (*Pacifasticus*

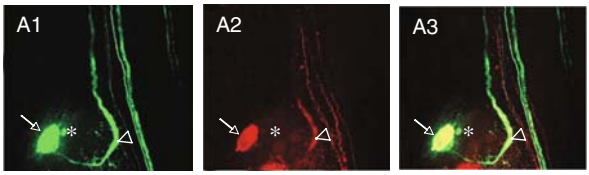

Fig. 13.1 Colocalization of tyrosine hydroxylase-like immunoreactivity and serotonin-like immunoreactivity in the L-cell of the blue crab, *Callinectes sapidus*. (A1) THli in the L-cell (*arrow*) and a second small cell body (*asterisk*; detected with a mouse monoclonal primary antibody and Alexa 488 goat anti-mouse secondary antibody). Note that the axon of the L-cell is constricted as it passes through the commissural ganglion, but that it then widens (*arrowhead*) after entering the circumesophageal connective to ascend to the brain (see text and Fort et al. 2004 for overall L-cell structure). (A2) 5HTli in the same preparation shown in *a1*. 5HTli was observed in the L-cell soma (*arrow*) and in the axon ascending in the connective (*arrowhead*; visualized with rabbit polyclonal antibody and Alexa 546 goat anti-rabbit secondary antibody). It was not detected in the initial segment of the L-cell. 5HTli was not seen in the small THli neuron (*asterisk*) or in other THli fibers, supporting the deduction that icolocalization of markers does not reflect an artifact of marker 'bleedthrough'. *Calibration bar* = 100 μm, applies to *A1–A3*. (*See* Color Plate 15)

leniusculus) colocalization with TH-like immunoreactivity was demonstrated in whole mount ganglia. (3) In double labeling whole mount experiments conducted on the prawn, *Macrobrachium rosenbergii*, Sosa et al. (2002) found serotonin and TH-like immunoreactivities in a large CG neuron that exhibited the anatomical features of the L-cell.

We used double-labeling immunohistochemical methods to assess dopamine/serotonin colocalization in the L-cells of the blue crab *Callinectes sapidus* (García et al. 2007). Previous studies using antibodies to dopamine and TH demonstrated the presence of DA in the *Callinectes* L-cell (Wood and Derby 1996; Fort et al. 2004). When double-labeling (TH and serotonin) experiments were performed, serotonin-like immunoreactivity was observed in the L-cell soma (Fig. 13.1). As observed in other species (Cournil et al. 1984; Tierney et al. 1999; Sosa et al. 2002) the serotonin-like immunoreactivity was less intense than that observed for TH.

13.5 Cholinergic/GABAergic Interneurons in *Aplysia*

The neuronal network that controls consummatory feeding behaviors in the marine mollusk *Aplysia* has been the subject of intensive study aimed toward disclosing principles of motor system organization and plasticity (Kupfermann 1974a,b; Elliott and Susswein 2002; Cropper et al. 2004). In their original identification of interneurons that could contribute to initiating patterned activity in the feeding motor system of *Aplysia*, Susswein and Byrne (1988) designated one

such cell B34[1] (see also Hurwitz et al. 1994). Subsequent investigations demonstrated that B34 possesses multi-action synaptic capabilities, exerting excitatory synaptic connections on certain follower neurons and inhibitory actions on others (Hurwitz et al. 1997). The observation that its excitatory synaptic actions were blocked by hexamethonium (10^{-4} M) led to the proposal that acetylcholine acts as the neurotransmitter of B34 (Fig. 13.2a; from Hurwitz et al. 2003). Subsequently, B34 was found to contain GABA-like immunoreactivity and its rapid inhibitory IPSPs to other targets were shown to be blocked by picrotoxin (Fig. 13.2b; from Jing et al. 2003). It was therefore proposed that B34 could be

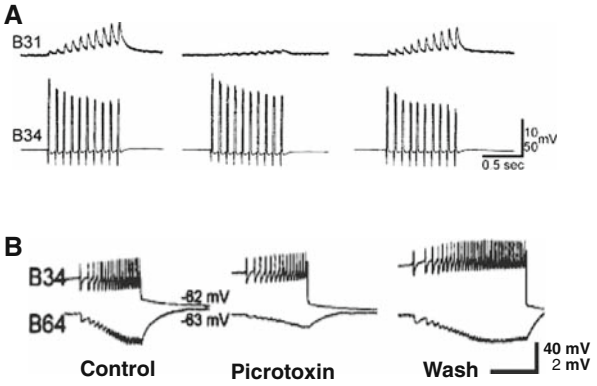

Fig. 13.2 Pharmacological observations indicating cholinergic and GABAergic signaling by B34, an interneuron in the feeding motor circuitry of *Aplysia*. (A) (*reprinted from Hurwitz et al*. 2003) *left*: firing B34 (*lower record*) for 1 s at a frequency of 10 Hz evoked a train of facilitating EPSPs in identified postsynaptic neuron B31 (*upper record*). *A, middle*: the EPSPs were blocked by hexamethonium (5×10^{-4} M). *A, right*: the effect of hexamethonium was reversed following washout of the drug. (B) (*reprinted from Jing et al*. 2003) *left*: firing B34 (*upper record*) evoked a train of IPSPs in identified postsynaptic neuron B64 (*lower record*). *B, middle*: the IPSPs were diminished by picrotoxin (1 mM). *B, right*: the effect of picrotoxin was reversed following washout of the drug. *A* and *B* were both performed in a raised divalent saline that attenuates polysynaptic signaling. In *B*, the postsynaptic membrane potential was pre-set to a level 15 mV more depolarized than rest (*V*m indicated for each neuron in *left* panel) to enhance IPSP amplitudes

[1] The neuronal network that generates *Aplysia* consummatory behaviors is located primarily in the buccal and cerebral ganglia. These ganglia have a bilaterally symmetrical organization and all neurons discussed in this article occur as pairs, one in each hemiganglion, unless otherwise noted. Cell nomenclature denotes the ganglion in which the cell body is located (Buccal in the case of B34). Numerals convey nominal information only, and do not specify neuron structure, function, or phenotype.

using the two distinct neurotransmitters, ACh and GABA respectively, to exert its rapid excitatory and inhibitory synaptic actions (Jing et al. 2003).

The divergent synaptic actions of B34 can be interpreted in the context of its proposed participation in feeding motor programs (Hurwitz et al. 1997; Jing and Weiss 2001; Jing et al. 2003; Cropper et al. 2004). Such motor programs are always initiated by a protraction of the tongue-like radula that is followed by a phase of radula retraction. B34 fires during the phase of radula protraction and its synaptic actions include excitation of protraction interneurons, and inhibition of interneurons that generate the antagonistic movement of retraction. Its cholinergic signaling is therefore thought to enhance and prolong radula protraction, while its GABAergic inhibitory signaling delays the onset of radula retraction. The efficiency of controlling two sequential phases of a motor program with a single neuron can be readily appreciated, as it will ensure that motor signals specifying the two antagonistic movements do not overlap. Any advantage that may be conferred by implementing such control with two distinct neurotransmitters, however, remains to be determined (see discussion below, under Overview and Future Directions).

13.6 Dopaminergic/GABAergic Interneurons in *Aplysia*

The neuronal network that controls feeding in *Aplysia* can be configured to perform multiple consummatory behaviors. Such multifunctionality is achieved via recruitment of particular interneurons that specify distinct motor patterns (Kupfermann and Weiss 2001). Two such interneurons, B20 and B65, were initially identified on the basis of their ability to elicit coordinated rhythmic motor programs from the feeding network (Teyke et al. 1993; Kabotyanski et al. 1998). Aldehyde fluorescence histology showed B20 and B65 to be catecholaminergic and pharmacological data supported their dopaminergic signaling (Teyke et al. 1993; Kabotyanski et al. 1998).

Subsequently, a survey of GABA-immunoreactive neurons in the central nervous system of *Aplysia* revealed GABAli cells with morphological similarities (size, shape, position, projections) to B20 and B65 (Díaz-Ríos et al. 1999). These observations prompted a systematic investigation in which GABA-catecholamine colocalization was tested using four independent protocols: (1) nerve backfill combined with GABAli, (2) FaGlu histochemistry combined with GABAli, (3) THli combined with GABAli, and (4) electrophysiological identification combined with GABAli (Díaz-Ríos et al. 2002). This study demonstrated that colocalization of GABA and DA markers was limited to five neurons in the entire CNS of *Aplysia*; the paired B20 cells, the paired B65 cells, and one unpaired neuron that has not as yet been identified

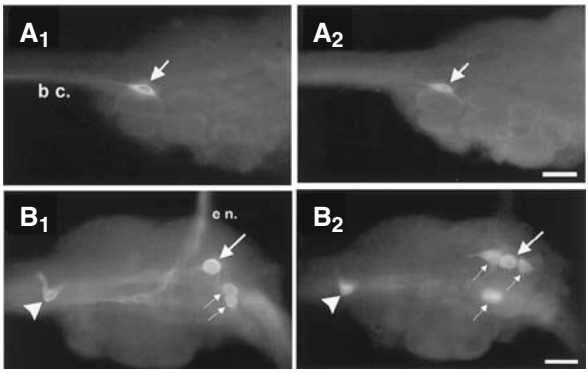

Fig. 13.3 Colocalization of THli and GABAli in neurons B20 and B65 of *Aplysia californica* (reprinted with permission from Díaz-Ríos et al. 2002). (A1): THli was observed in a single neuron, B20 (*arrow*), on the rostral surface of each buccal hemiganglion (only the left hemiganglion is shown). (A2): GABAli in the same preparation as *a1*. GABAli was also localized to the B20 neuron (*cf*. arrows in *a1* and *a2*). Scale bar: 40 μm applies to *a1* and *a2*. (B1): THli was observed in four neurons on the caudal surface of each buccal hemiganglion (the right hemiganglion is shown); one unpaired cell (*arrowhead*) near the buccal commissure and three cells (*arrows*) in the lateral region of the ganglion. (B2): GABAli in the same preparation as *B1*. GABAli was localized to the unpaired neuron (*cf*. *arrowheads* in *b1* and *b2*). It was also present in four lateral neurons, one of which corresponded to B65, the THli labeled in panel *b1* (*cf*. large arrows in *b1* and *b2*). Scale bar: 40 μm applies to *b1* and *b2*

(Fig. 13.3; from Díaz-Ríos et al. 2002). The presence of GABAli in B65 was confirmed independently by Jing et al. (2003).

Following the demonstration of DA-GABA colocalization in B20 and B65, experiments were performed aimed toward identifying the neurotransmitters mediating their synaptic signaling (Due et al. 2004; Díaz-Rios and Miller 2005, 2006). The rapid EPSPs from B65 and B20 to specific followers were occluded by dopamine, but not GABA, and blocked by the dopamine antagonist sulpiride (Due et al. 2004; Díaz-Ríos and Miller 2005). It was therefore proposed that these rapid EPSPs were mediated by dopamine. GABA, acting through $GABA_B$-like receptors, was shown to modulate the rapid dopaminergic EPSPs in a target specific manner (Díaz-Ríos and Miller 2005, 2006). To date, there is no evidence for inhibitory signaling or rapid GABAergic PSPs originating from B20 or B65 (*cf*. B34 above).

The GABAergic modulation of signaling by B20 was further examined in studies aimed toward disclosing the contributions of GABA to various forms of synaptic plasticity (Svensson et al. 2004; Díaz-Ríos and Miller 2006). GABA was found to potentiate inward currents produced by dopamine on specific postsynaptic targets (Svensson et al. 2004) and GABA was proposed to potentiate three forms of synaptic plasticity; short-term potentiation (Svensson et al.

2004), facilitation, and summation (Díaz-Ríos and Miller 2006). In all cases, data supported a postsynaptic action of GABA that was mediated via $GABA_B$-like receptors.

In sum, the available data indicate that GABA and DA are colocalized in a limited number of neurons that are highly influential in promoting and shaping the feeding motor programs of *Aplysia*. Convergent and divergent rapid excitatory synaptic signaling from these neurons is mediated by dopamine. In the synapses that have been studied, GABA could modify the rapid dopaminergic signals via postsynaptic $GABA_B$-like receptors or presynaptic receptors. To gain additional support for GABA-DA colocalization and cotransmission, we have begun to explore whether similar patterns occur in related mollusks (Fig. 13.4). On the rostral surface of the buccal ganglion of *Dolabrifera dolabrifera*, another member of the Aplysiidae family, GABA-DA colocalization was observed in a bilateral pair of neurons (one shown in Fig. 13.4a1–3) near the buccal commissure. The size, position, and branching pattern of these cells suggest that they correspond to the B20 interneurons of *Aplysia*. A second pair of GABA-DA neurons was observed more laterally and closer to the caudal surface of the *Dolabrifera* buccal ganglion (one shown in Fig. 13.4b1–3), in a position corresponding to the B65 interneuron of *Aplysia*.

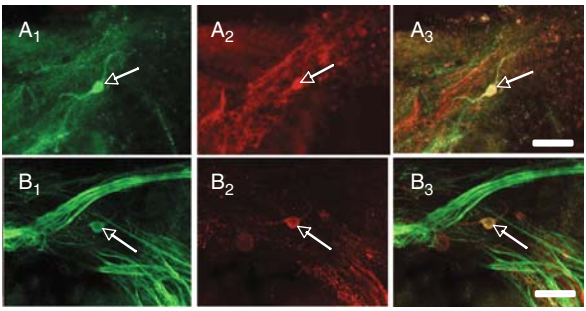

Fig. 13.4 Colocalization of TH-like immunoreactivity and GABA-like immunoreactivity in the buccal ganglion of *Dolabrifera dolabrifera*. (*A1*) A single neuron (*arrow*) on the rostral surface was marked with an antibody against tyrosine hydroxylase (mouse monoclonal; Alexa 488 goat anti-mouse secondary). (*A2*) When the same preparation was processed for GABA-like immunoreactivity (rabbit polyclonal; Alexa 546 goat anti-rabbit secondary), the same neuron (*arrow*) was marked. (*A3*) The labeled neuron (*arrow*) appears yellow in an overlay of panels *A1* and *A2*. *Calibration bar*: 100 μm applies to all *A* panels. (*B1*) A neuron (*arrow*) in the lateral region of the caudal surface of each buccal hemiganglion (only the right is shown) was marked with an antibody against TH. (*B2*) When the same preparation was processed for GABA-like immunoreactivity, the same neuron (*arrow*) was marked. (*B3*) The labeled neuron (*arrow*) appears yellow in an overlay of panels *B1* and *B2*. *Calibration bar*: 100 μm applies to all *B* panels. (*See* Color Plate 16)

13.7 Overview

It is clear from this synopsis that large gaps exist in our present understanding of colocalization and cotransmission of classical neurotransmitters in invertebrates. Although the data reviewed allow few definitive conclusions to be drawn, they do permit some inferences and speculation that can guide further study.

1. The number of neurons in which two classical neurotransmitters are colocalized tends to be small in comparison to the number in which they are not. Far from being obligatory, the colocalization of two particular neurotransmitters appears to occur rarely in the invertebrate nervous systems that have been examined in greatest detail. In the case of GABA and dopamine in *Aplysia*, each neurotransmitter is present in more than fifty central neurons (Croll 2001; Díaz-Ríos et al. 1999), and yet their colocalization has only been observed in five cells (Díaz-Ríos et al. 2002). Similarly, while the central nervous systems of decapod crustaceans contain up to one hundred serotonergic and dopaminergic neurons (Beltz and Kravitz 1983; Tierney et al. 2003), the overlap of these two systems has only been reported to occur in the L-cell.

2. It is not possible to extract rules concerning which classical neurotransmitters are more or less likely to be paired as cotransmitters. In the limited number of examples described, GABA was paired with DA and ACh, ACh was paired with GABA and serotonin, serotonin was paired with ACh and dopamine, and dopamine was paired with serotonin and GABA. It may be anticipated that other pairings will emerge as additional markers for classical neurotransmitters become available (see following text).

3. Classical neurotransmitter colocalization can occur in a variety of neuron types. This article has described the coexistence of classical neurotransmitters in neurosecretory cells, proprioceptors, and interneurons within a central pattern generator circuit. Interestingly, although neuropeptide cotransmitters are utilized extensively by the motor neurons of invertebrates, the presence of multiple classical transmitters in motor neurons has not yet been described.

13.8 Future Directions

Each of the neurons considered in this chapter participates in an intensively investigated motor circuit. In most instances, direct postsynaptic targets are known, and the contributions of these neurons to motor pattern generation or regulation can be evaluated. These systems should therefore present exceptional opportunities for studying the functional contributions of classical neurotransmitter colocalization and cotransmission to synaptic integration and circuit operation.

1. How can classical cotransmitters broaden the signaling capacity of individual neurons? Cotransmitters can expand signaling in the temporal or spatial domains. Temporally, classical cotransmitters may enable a neuron to influence a particular postsynaptic target on multiple timescales. Spatially, divergent

synaptic cotransmission can increase the number of postsynaptic cells that a single neuron can influence. Each of these forms of enhanced signaling depends upon the nature and distribution of the receptors that are present on the neurons that receive signals from a cotransmitter-containing neuron (Marder et al. 1995; Marder 1999). In principle, given a sufficient variety of receptors, they could be achieved using a single multi-action neurotransmitter (see Gardner and Kandel 1972; Katz and Frost 1995).

A complementary model of cotransmitter function proposes that multiple released substances may activate common or convergent signaling pathways with distinct efficacies (Brezina and Weiss 1997a,b). As a result of their varying capabilities to stimulate these pathways, combinations of coactive transmitters can achieve a range or precision of signaling that could not be accomplished by any of the transmitters acting alone. Notably, this hypothesis specifies a computational benefit imparted by cotransmitters that may not be readily achieved by additional classes of postsynaptic receptors.

Finally, cotransmission by classical neurotransmitters may enable the signaling of a neuron to be precisely regulated by its own previous activity. In contrast to peptide cotransmitters, which are typically thought to be packaged in large dense core vesicles that possess distinct release properties, impulse-mediated cotransmission of classical neurotransmitters could be envisioned to produce specific stoichiometries of signaling molecules within the synaptic cleft. One consequence of such cotransmission could be the modification of short-term synaptic plasticity during repetitive firing (see Svensson et al. 2004; Díaz-Ríos and Miller 2006).

2. Do classical cotransmitters enhance the ability of individual neurons to regulate motor circuits? Several of the neurons considered in this article are found at the boundaries between sensory and motor systems. They are thus effectively positioned to control motor patterns via signals that exert multiple actions on central pattern generator circuits. It has been proposed that such motor system regulation can be achieved via two general architectures. When motor system regulation is imposed by neurons that are not *sensu stricto* participants in the CPG, it is termed extrinsic (Kupfermann 1979; Morgan et al. 2000). When it derives from neurons that are themselves elements of the CPG (Katz and Frost 1996), or from motor neurons (Cropper et al. 1987), it is designated intrinsic. The invertebrate neurons that are proposed to utilize multiple classical cotransmitters embody both of these major regulatory motifs. While the L-cell and the GPR neurons can exert strong effects on the motor circuits that they regulate, their activity is not required for the motor patterns to be expressed. The interneurons of the *Aplysia* feeding network (B34, B20, and B65), on the other hand, are more deeply embedded within the buccal CPG that they regulate.

Consistent with its potential contributions to synaptic signaling considered above, cotransmission by colocalized classical neurotransmitters could contribute to motor system regulation in both temporal and spatial domains. Temporally, the consequence of signaling on multiple timescales to CPG

operation was emphasized by Getting (1989), who recognized how such signals could enable individual interneurons to influence multiple phases of motor activity. Spatially, cotransmission may enable an individual interneuron to efficiently achieve coactivation of combined populations of motor neurons, each of which, under other conditions, may be individually activated by interneurons that only utilize one neurotransmitter. Finally, direct synchronous excitation and inhibition of motor neurons whose coactivation is inconsistent with adaptive motor patterns could produce a level of precision in phase timing and phase transitions that could not be achieved with interposed interneurons.

13.9 Conclusions

Clearly, much investigation will be required to validate and clarify the instances of classical neurotransmitter colocalization and cotransmission that have been described in this chapter. Recent genomic and neuromic initiatives with invertebrate model organisms promise to provide additional markers that will facilitate demonstrations of colocalized classical neurotransmitters in these systems (Moroz et al. 2006; Schulz et al. 2007 see also www.NeuronBank.org). Emerging technologies and approaches should also provide tools to explore important cell biological questions, including transmitter biosynthesis, sorting, compartmentalization, release, and uptake in invertebrate neurons that contain more than one classical neurotransmitter (Fuller et al. 1998; Anderson and Ewing 1999; Martí et al. 2006, 2007).

Even in the simple systems considered here, it may be appreciated how the adaptive operation of neural networks can be enhanced if multiple signals are implemented at precisely the same time (synchrony) or in precisely the same place (convergence). In our present understanding of information transfer in nervous systems, the synapse represents the highest level of both temporal and spatial precision, and synaptic cotransmission can clearly achieve levels of synchrony and convergence that could not be accomplished by two independent neurons utilizing a single neurotransmitter. Thus, although we can speculate about benefits that colocalization and cotransmission of classical neurotransmitters could confer upon a neuron's contribution to circuit function, the pressures and constraints that would lead to this resolution in specific neurons, and not in others, remain unknown. In view of nature's inherently conservative approach to problem-solving in neural systems, however, it may be expected that analogous computational challenges were met with similar resolutions in more complex nervous systems, including our own brains.

Acknowledgments Supported by: the National Institutes of Health: NIGMS MBRS: GM-08224 and NCRR RCMI G12 RR03051; the National Science Foundation: DBI-0115825.

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