IDENTIFICATION AND EFFECTS OF NEURAL TRANSMITTERS IN INVERTEBRATES

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INTRODUCTION

This review treats almost exclusively of investigations that have been published over the last three years (for work prior to that period see 1). Preference is given to data obtained from identified neurons, in particular, from neurons for which the transmitter has been evaluated biochemically as well as electrophysiologically and pharmacologically. Finally, only those synaptic studies that deal directly with the problems of transmitter pharmacology have been considered.

The first half of this review deals with recent advances in single-cell neurochemistry. Biochemical and histochemical work concerned with the identification of transmitters in identified neurons is summarized in Table 1, and is followed by a brief consideration of some issues raised by the table and by other recent work in this field. The second half of this review covers electrophysiological and pharmacological studies that clarify the role of transmitters or putative transmitters at some identified junctions, giving preferential treatment to experimental preparations and problems that have received rather extensive attention.

NEUROCHEMISTRY

One of the major advances in the field of invertebrate neurochemistry is the development of sophisticated techniques for the measurement of putative transmitter substances in single identified neurons. These techniques have been used for two main purposes: (*a*) to identify transmitter candidates, and (*b*) to study the dynamic aspects of transmitter synthesis, mobilization, storage, and release in identified neurons. These techniques have been used to determine endogenous levels (in picomole quantities) of γ-aminobutyric acid (GABA) (2), dopamine (3), acetylcholine (ACh) (4), octopamine (5), histamine (6, 7), glutamate (2, 8), and serotonin (5-HT)

(3, 7). In addition, the enzymes responsible for the biosynthesis of putative transmitters can be assayed, either directly [(choline acetyltransferase (9–12), aromatic amino acid decarboxylase (13–15), or glutamic acid decarboxylase (16–19)] or indirectly by measuring the incorporation of radioactive precursors into putative transmitters, using uptake from the bath (20, 21), or by injecting radioactively labeled precursors into neuronal somata (22, 23) or axons (24).

Much recent work has been done on the characterization of transmitter biosynthetic enzymes in invertebrate nervous tissue (9, 11–13, 17, 25–27) and on the measurement of endogenous levels of putative transmitters in ganglia and connectives of many animals (3, 4, 6, 11, 28–32). The emphasis of this review is on single cells, and Table 1 is a summary of presently available biochemical and histochemical evidence on identified neurons.

In the past, biochemical analyses have focused on finding neurons that contain considerably higher concentrations of a putative transmitter than do other neurons (1, 2, 4, 61). This approach remains valuable for suggesting new transmitter candidates and has brought histamine and octopamine into consideration as possible transmitters in invertebrate neurons. Weinreich, Weiner & McCaman (6) assayed histamine in single *Aplysia* neurons and found that although there were low levels of histamine in several of the neurons they assayed, two cerebral ganglia neurons contained considerably higher histamine concentrations. These neurons are promising candidates for histamine-releasing neurons, and this information facilitates a physiological search for a histamine-mediated monosynaptic connection. Likewise, octopamine-containing neurons have been found in *Aplysia* ganglia (5) and in the second root of the thoracic connective in *Homarus* (66) (see last section of this paper for a discussion of the currently available literature concerning octopamine function in arthropod systems).

Examination of Table 1 shows that in single neurons whose transmitter is known or suspected low quantities of many other putative transmitters can be detected. For example, R2 in *Aplysia* contains about 25 pmol of ACh (4) (thought to be its transmitter) but also contains about 0.15 pmol of histamine (6) and 0.43 pmol of octopamine (5). This is no new phenomenon. For example, Kravitz & Potter (61) found low levels of GABA in sensory and excitatory axons in *Homarus*. In this and other similar cases the concentration of the putative transmitter substance in the neurons thought to employ it as a transmitter was about 100-fold higher than in the neurons not thought to use it as transmitter.

Two recent studies have sought to refocus our attention on these low levels of multiple transmitter molecules. Hanley et al (58) report that the metacerebral giant cell in *Helix*, which uses 5-HT as a transmitter, also contains significant choline acetyltransferase activity. Hanley & Cottrell (57) found 0.1 ng of ACh in the soma of the metacerebral cell, which also contains about 1.0 ng of 5-HT. Brownstein et al (7) present measurements of many transmitter substances in several *Aplysia* neurons.

Two questions can be raised about these low levels of multiple putative transmitter substances. First, are they possibly experimental artifacts? Virtually all of this work has been done on hand-dissected neurons, which certainly contain an un-

Table 1 Single cell transmitter chemistry[a, b]

Animal	Cell	Suspected transmitter	Substance assayed	Amount per cell	Concentration	Biosynthetic capability	Physiological corroboration
Leech	retzius	5-HT	5-HT	3.8×10^{-10} g (34)	6.0 mM (34)	AAD—25.6 pmol DA/cell/hr (14)	(33)
			5-HT	2.5 pmol (3)		incorporation of ^{14}C-trp and ^{14}C-5-HTP into ^{14}C-5-HT (20)	
Aplysia	R2	ACh	ACh	25.7 pmol (4)	0.39 mM (4)	ChAc—2.11 nmol ACh/cell/hr (4)	
			choline	11.0 pmol (4)		ChAc—3.0 nmol ACh/cell/hr (9)	
						~ 80% conversion of injected ^{3}H-choline to ^{3}H-ACh (22–24, 36)	
			oct	0.43 pmol (5)	0.0025 mM (5)	AAD—440 pmol DA/cell/hr (13)	
			5-HT		0.018 mM (7)	1.4% conversion of injected ^{3}H-5-HTP to ^{3}H-5-HT (37)	
			HA	0.15 pmol (6)	0.002 mM (6)		
					0.003 mM (7)		
			asp	1746 pmol (38)	25.0 mM (38)		
			glu	86 μmol/gprot (8)			
			gln	39 μmol/gprot (8)			
Aplysia	R14	?	oct	3.66 pmol (5)	0.15 mM (5)	AAD—144.7 pmol DA/cell/hr (13)	
			HA	0.12 pmol (6)	0.005 mM (6)		
			HA		0.007 mM (7)		
			5-HT		0.034 mM (7)		
			asp	586 pmol (38)	27.0 mM (38)		
			glu	34 μmol/gprot (8)			
			gln	21 μmol/gprot (8)			
Aplysia	R15	"	asp	833 pmol (38)	47.0 mM (38)	AAD—5.20 pmol DA/cell/hr (13)	
			glu	64 μmol/gprot (8)			
			gln	24 μmol/gprot (8)			

Table 1 *(Continued)*

Animal	Cell	Suspected transmitter	Substance assayed	Amount per cell	Concentration	Biosynthetic capability	Physiological corroboration
Aplysia	L2-L6	?	oct	1.04 pmol (5)	0.046 mM (5)	AAD—152-369 pmol DA/cell/hr (13)	
			asp	1152 pmol (38)	28.0 mM (38)		
			glu	44 μmol/gprot (8)			
			gln	24 μmol/gprot (8)			
Aplysia	L7	?	oct	1.46 pmol (5)	0.065 mM (5)	AAD—135.8 pmol DA/cell/hr (13)	(39)
			asp	771 pmol (38)	34.0 mM (38)		
			glu	94 μmol/gprot (8)			
			gln	32 μmol/gprot (8)			
Aplysia	L10	ACh	ACh	3.8 pmol (4)	0.35 mM (4)	ChAc—2.16 nmol ACh/cell/hr (4)	(42-44)
			choline	9.9 pmol (4)		ChAc—1.8 nmol ACh/cell/hr (9)	
						85% conversion of injected ^{3}H-choline to ^{3}H-ACh (23. 41)	
						firing-dependent, dependent release of radioactivity ^{3}H-choline (41)	
			oct	0.11 pmol (5)	0.014 mM (5)	AAD—218 pmol DA/cell/hr (13)	
Aplysia	L11	ACh	ACh	6.2 pmol (4)	0.33 mM (4)	ChAc—1.8 nmol ACh/cell/hr (4)	
			choline	13.3 pmol (4)		ChAc—2.0 nmol ACh/cell/hr (9)	
						71% conversion of injected ^{3}H-choline to ^{3}H-ACh (23)	
			oct	1.6 pmol (5)	0.009 mM (5)	AAD—279 pmol DA/cell/hr (13)	
			5-HT		0.011 mM (7)		
			HA		0.005 mM (7)		
			asp	1237 pmol (38)	44.0 mM (38)		
			glu	65 μmol/gprot (8)			
			gln	23 μmol/gprot (8)			

Table 1 *(Continued)*

Aplysia	L13		oct	0.19 pmol (5)	0.023 mM (5)		
Aplysia	LD	ACh				84% conversion of injected ^{3}H-choline to ^{3}H-ACh (23)	
Aplysia	LDG_1	ACh				ChAc—630 pmol ACh/cell/hr (39) 86% conversion of injected ^{3}H-choline to ^{3}H-ACh (39)	(39)
Aplysia	LD_{HI}	ACh				82.5% conversion of injected ^{3}H-choline to ^{3}H-ACh (45)	(45, 46)
Aplysia	LB_{VC}	ACh				78% conversion of injected ^{3}H-choline to ^{3}H-ACh (45)	(45, 46)
Aplysia	RB	5-HT				9.7% conversion of injected ^{3}H-trp to ^{3}H-5-HT (23) 18.6% conversion of injected ^{3}H-5-HTP to ^{3}H-5-HT (37)	
Aplysia	RB_{HE}	5-HT				7.8% conversion of injected ^{3}H-trp to ^{3}H-5-HT (45)	(45, 46)
Aplysia	LPGC (left pleural giant)	ACh	ACh choline	30 pmol (4) 10.2 pmol (4)	0.34 mM (4)	ChAc—1.7 nmol ACh/cell/hr (4) ChAc—2.8 nmol ACh/cell/hr (9)	
			asp glu gln	2597 pmol (38) 66 μmol/gprot (8) 42 μmol/gprot (8)	0.34 mM (38)	AAD—180 pmol DA/cell/hr (13)	

Table 1 *(Continued)*

Animal	Cell	Suspected transmitter	Substance assayed	Amount per cell	Concentration	Biosynthetic capability	Physiological corroboration
Aplysia	LC2	?	HA	1.68 pmol (6)	0.476 mM (6)		
Aplysia	RC2	?	HA	0.99 pmol (6)	0.309 mM (6)		
Aplysia	C-1 (GCN)	5-HT	5-HT 5-HT	6.2 pmol (47)	0.94 mM (7)	AAD—2.3 nmol DA/cell/hr (47) 2.3% conversion of injected ^{3}H-trp to ^{3}H-5-HT (23) 24.7% conversion of injected ^{3}H-5-HTP to ^{3}H-5-HT (37)	(48)
			HA HA asp glu gln	0.18 pmol (6) 365 pmol (38) 36 μmol/gprot (8) 25 μmol/gprot (8)	0.012 mM (6) 0.014 mM (7) 34 mM (38)		
Aplysia	buccal cells	?	oct asp	0.09-1.3 pmol (5) 300-520 pmol (38)	0.012-0.39 mM (5) 49-90 mM (38)		
Tritonia	C-1	5-HT	5-HT	4.0 pmol (47)		AAD—620 pmol DA/cell/hr (47)	
Tritonia	PD-1	5-HT	5-HT	4.2 pmol (47)		AAD—377 pmol DA/cell/hr (47)	
Planorbis	left pedal giant neuron	DA	DA	5.4 pmol (49) fluorescence histochemistry (50)		synthesis of ^{3}H-DA from ^{3}H-tyr (51)	(52)
Planorbis	GSC	5-HT	5-HT	fluorescence histochemistry (50)			

Table 1 *(Continued)*

Helix	GSC (metacerebral giant)	5-HT	5-HT 5-HT	fluorescence histochemistry (53) 1.1 ng (56)	AAD—21 pmol DA/cell/hr (15) synthesis of ^{14}C-5-HT from ^{14}C-5-HTP (56) AAD—26 pmol DA/cell/hr (58)	(54)
			ACh	0.1 ng (57)	ChAc—20–22 pmol ACh/cell/hr (58)	
Helix	buccal 0–6	?			ChAc—18–31 pmol ACh/cell/hr (15)	
Helix	buccal 1, 2				AAD—11 pmol DA/cell/hr (15)	
Helix	cerebral 7–12	?			ChAc—9–26 pmol ACh/cell/hr (15)	
Helix	p. visceral 13–26	?			ChAc—22–99 pmol/ Ach/cell/hr (15)	
Helix	p. visceral 5–14				AAD—9–52 pmol DA/cell/hr (15)	
Helix	pedal 27–36	?			ChAc—11–127 pmol ACh/cell/hr (15)	
Helix	pedal 15–20				AAD—8–24 pmol DA/cell/hr (15)	
Schistocerca	common inhibitor	GABA			GAD—12.9 pmol GABA/cell/hr (19)	
Chortoicetes	common inhibitor	GABA			GAD—14.4 pmol GABA/cell/hr (19)	
Chortoicetes	anterior inhibitor	GABA			GAD—19.3 pmol GABA/cell/hr (19)	
Chortoicetes	posterior inhibitor	GABA			GAD—22.5 pmol GABA/cell/hr (19)	

Table 1 *(Continued)*

Animal	Cell	Suspected transmitter	Substance assayed	Amount per cell	Concentration	Biosynthetic capability	Physiological corroboration
Romalae	DUMETI	oct				synthesis of ^{3}H-oct from ^{3}H-tyr (59)	(60)
Homarus	I2	GABA	GABA	79 pmol (2)	13.4 mM (2)	^{14}C-GABA synthesized from ^{14}C-glu (20)	
			glu	103 pmol (2)	14.9 mM (2)		
Homarus	I1	GABA	GABA	27 pmol (2)			(110) stimulation-produced release of GABA (40)
Homarus	I3	GABA	GABA	44 pmol (2)	14.6 mM (2)		
			glu	96 pmol (2)	20.6 mM (2)		
Homarus	inhibitor, opener crusher claw	GABA					*stimulation*-produced release of GABA (40)
Homarus	inhibitor, opener cutter claw	GABA					*stimulation*-produced release of GABA (40)
Homarus	inhibitor, walking leg opener	GABA	GABA	1.4 nmol/cm axon (61)	99 mM (61)		
Homarus	inhibitor, walking leg closer	GABA	GABA	1.5 nmol/cm axon (61)	110 mM (61)	GAD—90 pmol GABA/cm axon/hr (18)	
			GABA		49.5 mM (55)		
			glu		31.5 mM (55)		
			asp		149.5 mM (55)		
Homarus	inhibitor, walking leg flexor	GABA	GABA	0.22 nmol/cm axon (61)	108 mM (61)		

Table 1 *(Continued)*

Homarus	M6/M7	glu	glu	85 pmol (2)	18.3 mM (2)		
Homarus	excitor, walking leg opener	glu	GABA glu asp	90 pmol/cm axon (61)	0.64 mM (61) 41.6 mM (55) 131.9 mM (55)		
Homarus	large excitor, closer walking leg	glu	GABA	20 pmol/cm axon (61)	0.83 mM (61)		
Homarus	small excitor, closer walking leg	glu	GABA	6.9 pmol/cm axon (61)	0.58 mM (61)		
Homarus	large excitor, walking leg bender	glu	GABA	14 pmol/cm axon (61)	1.0 mM (61)		
Homarus	small excitor, walking leg bender	glu	GABA	9.5 pmol/cm axon (61)	0.88 mM (61)		
Homarus	abdominal stretch receptors	ACh	ACh	1.9 pmol/μg prot (11) 2.9 pmol/cm axon (11)	0.4–0.6 mM (11)	ACh synthesis (20) ChAc—17.1 pmol ACh/cm axon/hr (21, 11)	(21)
Homarus	medial giant fibers	?	GABA	40 pmol/cm axon (61)	0.68 mM (61)		
Homarus	lateral giant fibers	?	GABA	50 pmol/cm axon (61)	0.59 mM (61)		
Homarus	thoracic ganglion 2nd root cells	oct	oct	~10 pmol (66)	~0.1 mM (66)	biosynthesis from ^{3}H-tyrosine and ^{3}H-tyramine (66)	high K^{+} induced ^{3}H-oct efflux (67)

Table 1 *(Continued)*

Animal	Cell	Suspected transmitter	Substance assayed	Amount per cell	Concentration	Biosynthetic capability	Physiological corroboration
Panulirus	large commissural ganglion cell	DA	DA	fluorescence histochemistry (63)	^{3}H-DA synthesis from ^{3}H-tyr (63)		
Panulirus	stomatogastric motor neurons PD, VD, GM, LPG	ACh				ChAc—5–30 pmol ACh/cell/hr (65)	(64, 65)
Carcinus	large commissural ganglion cell	DA	DA	fluorescence histochemistry (62)			
Cancer	pooled walking leg excitors	glu	glu asp GABA		53 mM (31) 196 mM (31) 3.1 mM (31)		
Cancer	pooled walking leg inhibitors	GABA	GABA glu asp		46 mM (31) 47 mM (31) 168 mM (31)		

[a] In the interests of brevity, results of assays that were negative were omitted, although they would in some cases have added interesting information.

[b] Abbreviations used in this table: AAD, aromatic amino acid decarboxylase; ACh, acetylcholine; asp, aspartate; ChAc, choline acetyltransferase; DA, dopamine; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; gln, glutamine; glu, glutamate; HA, histamine; 5-HTP, 5-hydroxytryptophan; oct, octopamine; tyr, tyrosine; trp, tryptophan. Abbreviations used in the name of cell column are those used by the quoted authors.

known amount of glial and likely even neuronal contamination. With increasingly sensitive assays, the small amounts of substances contributed by contaminating tissue become more significant. In injection studies, leakage could be a problem. Second, even if low levels of substances are actually present in the neurons involved, are they physiologically significant? Phrased differently, this question asks, how useful are biochemical techniques alone for transmitter identification? In reference to the work of Hanley et al (57, 58) the transmitter function of 5-HT has been confirmed by physiological studies (54) (see later section of this paper) whereas the ACh reported in this cell has not yet been shown to be utilized in a monosynaptic connection made by this neuron. These data point out the caution that must be exercised when drawing conclusions from biochemical data alone, and stress the importance of combining physiological and biochemical techniques for transmitter identification.

A further examination of Table 1 shows that somata transmitter concentrations vary from about 0.3 mM for the large ACh-containing neurons of *Aplysia* (4) to about 15 mM for the large GABA-containing neurons of *Homarus* (2). The axonal concentrations of GABA in the latter cells are even higher: about 100 mM (61). It is interesting that the axons of the assayed excitatory motor neurons of lobster had GABA concentrations of almost 1 mM (61) i.e. a concentration higher than that of ACh in cholinergic cells [e.g. molluscan somata (4) or the *Homarus* stretch receptor axons (11)]. Thus it appears that the absolute concentration of these substances is not a useful indicator of transmitter function unless extensive data on other cells and other transmitters are available for comparison.

Aromatic amino acid decarboxylase (AAD) is involved in the biosynthesis of dopamine, octopamine, and 5-HT in invertebrate nervous tissue. However, Weinreich, Dewhurst & McCaman (13) showed that all *Aplysia* cells assayed contained significant levels of AAD. This fact was exploited by Goldman & Schwartz (37) in an interesting attempt to study the specificity of transmitter packaging and storage in cholinergic and serotoninergic neurons in *Aplysia*. These authors found that after injecting ^{3}H-5-HTP, the immediate precursor of 5-HT and substrate for aromatic amino acid decarboxylase, they detected significant amounts of 5-HT in the cholinergic neuron R2. However, they found that whereas the ^{3}H-5-HT formed in serotonin-containing neurons was transported into the axon, and was associated with particulate fractions (presumably synaptic vesicles), the ^{3}H-5-HT formed in R2 was apparently not packaged and transported. From these data the authors argued that in addition to specificity in transmitter biosynthetic capability there is also specificity in packaging and transport mechanisms. It would have been interesting to have the results of a similar experiment done on a cholinergic neuron that had been depleted of ACh, by one means or another, to determine whether the cholinergic neuron was capable of storing and transporting 5-HT under conditions where ACh was not present and synthesized.

Biochemical techniques have been used to study two additional processes directly involved in synaptic transmission: (*a*) release of the transmitter, and (*b*) uptake of both transmitter precursors and of the transmitters themselves as a mechanism of terminating the action of a synaptically released transmitter.

Demonstration of release of a given substance by synaptic activity is the most convincing proof that a substance is the neurotransmitter at a given junction, and this proof is most important for synapses where the suspected transmitter is a ubiquitous molecule, such as L-glutamate. Unfortunately, little new evidence has been forthcoming in this area since that reported and reviewed by Kravitz et al (68). The difficulty in obtaining such data is shown by the work of Koike et al (41) who injected ^{3}H-choline into L10, a known cholinergic neuron in *Aplysia* and then attempted to recover ^{3}H-ACh in amounts proportional to synaptic activity. The authors were able to demonstrate that released radioactivity is dependent on firing frequency and external calcium concentration, but were unable to recover the radioactivity as ACh, apparently because the acetylcholinesterase was incompletely blocked.

Thorough and interesting studies of uptake in invertebrate systems in recent years have been those by Evans on L-glutamate uptake in arthropod nervous tissue (69–71) and that by Schwartz et al (33) and Eisenstadt et al (72) on choline uptake and metabolism in *Aplysia* nervous tissue.

Schwartz et al (33) showed that choline uptake has two kinetic components: a high affinity uptake system with a K_m of about 2–8 μM and a low affinity system that does not saturate at 420 μM. Eisenstadt & Schwartz (36) followed this work with a very interesting study of ACh metabolism in R2 of *Aplysia.* They showed that choline concentrations in the soma limit the amount of somatic ACh synthesis, and that the proportion of radioactivity incorporated into ACh from choline is much greater when ^{3}H-choline is injected into the soma rather than taken up from the bath. Furthermore, the authors were able to measure the kinetics of ACh synthesis within the intact neuron, and found that their values were similar to those previously obtained for choline acetyltransferase in homogenates of *Aplysia* nervous tissue. Perhaps most disturbing of all for those who are using incorporation of radioactive precursors from the bath to study transmitter synthesis and mobilization, these authors found that ACh formed from choline in the bath turned over at a different rate from that formed from injected choline, which suggests that there are two pools of ACh within these neurons; one pool preferentially labeled when the choline enters the cell either by high affinity uptake or injection, and the other when the choline enters the cell by low affinity uptake. Under conditions of bath incubation the amount of choline entering the cell by low affinity uptake is considerable; if this situation is general, these considerations complicate the interpretation of experiments using bath-applied precursors.

GANGLIONIC SYNAPTIC TRANSMISSION IN INVERTEBRATES

Gastropod central neurons that, because of their large size, are so amenable to single-cell neurochemistry, have also proven to be a particularly satisfactory preparation for the study of receptor pharmacology and synaptic transmission. This experimental preparation has become even more valuable in recent years because of the identification of pairs of monosynaptically connected neurons, and, in some

cases, because of the identification of the transmitter used at the synapses made between these neuron pairs.

Acetylcholine

The analysis of the effects of ACh on gastropod neurons first revealed the complexity that seems to be typical of transmitter receptor systems in invertebrate ganglia (see 1, 77 for detailed and documented accounts). ACh, whether applied iontophoretically or released by activation of cholinergic neurons, was shown to elicit many different types of response. The response variety was shown to be due to the existence of three pharmacologically distinct ACh receptors, each of which mediates a selective change in membrane permeability. One receptor type mediates an increase in membrane permeability to Na (thereby causing an excitatory potential when activated); two others mediate inhibitory responses—one, resulting from an increase in permeability to Cl; the other, to K. These different receptor types have been shown to coexist on the same cell membrane, and, in such cases, ACh (whether applied synaptically or iontophoretically) elicits multicomponent responses.

A pharmacological comparison (see 77, 78) of the three types of ACh receptor on molluscan neurons with cholinergic receptors previously described in other neural tissues suggests that the molluscan receptor that mediates the increase in Na permeability most resembles the vertebrate receptor mediating the rapid excitatory postsynaptic potential (EPSP) of the sympathetic ganglion cells (both are blocked, for example, by hexamethonium as well as by curare). The molluscan receptor mediating the Cl-dependent inhibition, on the other hand, most resembles the receptor of the frog skeletal muscle (which, itself, mediates increases in Na and K permeabilities). These two receptors are much more sensitive to curare than to hexamethonium, this latter compound being completely without effect on the molluscan receptor mediating the Cl response.

The above two comparisons have recently been reinforced by findings with α-bungarotoxin (*B. multicinctus*) (78). This toxin has been shown to block quasi-irreversibly the frog end-plate potential, but to be ineffective in blocking the rapid EPSP (hexamethonium-sensitive) of the sympathetic ganglion. In *Aplysia* the same toxin was found to block the receptor mediating the Cl-dependent response (hexamethonium-insensitive) while having no effect on the receptor mediating the Na-dependent response (hexamethonium-sensitive) or on the receptor mediating the K-dependent response. Similarly selective actions of α-neurotoxins from *Dendroaspis viridis* were observed on the ACh responses in snail neurons (79).

The third cholinergic receptor of *Aplysia* neurons, that mediating an increase in K permeability, resembles no known vertebrate receptor (80), being unaffected by both curare and atropine, as well as by both nicotine and muscarine. It can, however, be selectively blocked by tetraethylammonium (TEA) and can be selectively activated by arecoline (see 77 for further characteristics). This pharmacological picture has recently been extended by the finding that it can also be selectively activated by a compound extracted from the venom of the conus snail (81). From data gathered on clam and *Aplysia* heart (see below) it appears that this same cholinergic receptor mediates inhibition in molluscan atrial cells.

Serotonin

The observation that 5-HT can depolarize and excite some snail neurons and hyperpolarize and inhibit others (see 1 for review) has been extended by the recent work of Gerschenfeld & Paupardin-Tritsch (82), which shows that six types of 5-HT response can be identified. Four of these responses are due to an increase in membrane conductance: Two (one rapid and one slow) are excitatory and result from an increase in Na conductance; two are inhibitory, one rapid, due to an increase in Cl conductance, and one slow, due to an increase in K conductance. In contrast, the last two of the six response types result from a *decrease* in membrane conductance; one is an excitatory response, due to a reduction in K conductance; the other, an inhibitory response due to decreases in both Na and K conductances.

The pharmacological analyses have shown that the responses that reflect increases in membrane conductance are due to the activation of four different types of 5-HT receptors. 7-Methyltryptamine blocks selectively the rapid Na-dependent response; 5-methoxygramine blocks only the K-dependent inhibitory response, whereas neostigmine blocks only the Cl-dependent inhibitory response. Curare blocks both the rapid Na-dependent response and the Cl-dependent inhibitory response, whereas bufotenine blocks the two Na-dependent excitatory responses (fast and slow) as well as the K-dependent inhibitory response (see 82 for further pharmacological characteristics). These results strongly suggest that at least the four responses resulting from an increase in membrane conductance are due to the activation of four distinct 5-HT receptors.

The two responses reflecting conductance decreases are less well understood. No specific antagonists have as yet been found. These responses, as well as another, nonserotoninergic, synaptically activated decrease in K conductance (83) observed in *Aplysia,* have ionic mechanisms that resemble those observed in the sympathetic ganglion by Weight & Votava (84). However, there has as yet been no evidence that these decreases in membrane conductance are associated with increases in either cAMP or cGMP, as are the potentials reflecting conductance decreases in the sympathetic ganglion (85, 86).

The relevance for synaptic transmission of this wide variety of serotonin response types has been revealed by the work of Cottrell & Macon (54) in *Helix pomatia* and by Gerschenfeld & Paupardin-Tritsch (48) in *Aplysia californica* using the biochemically defined serotoninergic neurons of the cerebral ganglia of these animals (see Table 1) and studying the responses to firing of these neurones in monosynaptically connected follower cells of the buccal ganglia. Analyses of the synaptic responses elicited by the serotoninergic neurons of *Aplysia* showed that at least four of the six response types can be elicited synaptically, and that these responses are affected by pharmacological agents in the same way as are the corresponding responses to iontophoretically applied 5-HT. These electrophysiological and pharmacological data show that the 5-HT biochemically detected in these neurons is indeed serving a transmitter function.

One action of 5-HT that is difficult to include in this rather extensive array of 5-HT receptors is that recently described by Shimahara & Tauc (87). These authors were able to cause, by repetitive stimulation of a nerve trunk, a long-lasting facilita-

tion of an EPSP evoked in the *Aplysia* giant cell by an identified, monosynaptically connected presynaptic neuron. This so-called heterosynaptic facilitation could be imitated by an iontophoretic application of 5-HT in the neuropile, with the 5-HT presumably reaching the presynaptic terminals of the EPSP-eliciting neuron. Both the facilitation produced by repetitive stimulation of the nerve trunk, as well as that caused by iontophoretically applied 5-HT, could be blocked by LSD. These authors concluded that afferent nerve stimulation causes a release of 5-HT into the presynaptic terminals and that the 5-HT causes a change in the presynaptic membrane that favors an increase in transmitter release when the presynaptic neuron is fired. This increased release is thus responsible for the long-term facilitation of the EPSP measured in the giant cell.

If such a serotoninergic effect takes place, it seems most probable that, in order to result in an increase in transmitter release, 5-HT would be causing a decrease in conductance of the presynaptic terminal membrane. Such decreases in membrane conductance by 5-HT were observed by Gerschenfeld & Paupardin-Tritsch (82) (see above), and might be a possible mechanism for heterosynaptic facilitation. However, the only responses that Gerschenfeld & Paupardin-Tritsch found that could be blocked by LSD were those mediated by a classical increase in membrane conductance; the two 5-HT elicited increases in membrane resistance that they demonstrated were unaffected by LSD, the drug used by Shimahara & Tauc to eliminate the heterosynaptic facilitation they observed. Thus, one must assume that if 5-HT is truly mediating the facilitatory effect via an increase in release from synaptic terminals, either a new receptor mechanism is involved, or it is in some way mediated via a more classical conductance *increase.*

Dopamine

A similar but somewhat less complex multireceptor system has emerged for the more recently established transmitter dopamine. Two types of dopamine response have been characterized in molluscan neurons (see 1, 77): one, a relatively rapid excitatory response due to an increase in cationic permeability; the other, a slow inhibitory response due to an increase in K permeability. Preliminary data of Carpenter & Gaubatz (88) suggest that certain dopamine responses might reflect an increase in membrane permeability to Cl ions.

In *Aplysia* it has been shown (see 77) that the excitatory dopamine response is much more readily desensitized than is the inhibitory response, and can be selectively blocked by curare and strychnine. The inhibitory response, on the other hand, can be blocked by ergometrine, which at higher doses also blocks the depolarizing response. Berry & Cottrell (52), studying the effects of iontophoretically applied dopamine in the central neurons of *Planorbis corneus,* showed that the excitatory and inhibitory responses they observed had the same pharmacological characteristics as the dopamine responses in *Aplysia.* They further extended the pharmacological analysis of dopamine receptors by finding a more specific blocking agent of the inhibitory response, 6-hydroxydopamine. They then studied the synaptic potentials elicited in monosynaptically connected follower cells in response to firing of the giant dopamine-containing neuron (49, 50), and demonstrated (1) that the response of a given follower cell to presynaptic stimulation was the same as that elicited by

iontophoretically applied dopamine, and (2) that the pharmacological effects observed on the synaptic responses were identical with those observed on the responses to iontophoretically applied dopamine. These experiments confirm the transmitter function of histochemically detected dopamine in these neurons.

Octopamine

Octopamine has been detected in individual neurons of *A. californica* (see Table 1) that do not contain dopamine and norepinephrine (5). Little is known of the effects of exogenously applied octopamine on possible target organs. One brief report (89) has been made on the observation that iontophoretically applied octopamine on certain cells of *Aplysia* elicits a hyperpolarization that seems to be due to an increase in K permeability. Until now, no connections have been established between the octopamine-containing neurons and follower cells, and until a battery of antagonists is developed, the confirmation of the transmitter role of octopamine might be delayed.

The above data yield a list of three rather firmly established transmitter substances in molluscan ganglionic synaptic transmission: ACh, 5-HT, and dopamine. In each of these cases it has been possible to study the effects of synaptically released as well as of the exogenously applied transmitter substance. It has been shown that for each of these transmitters, the molluscan neurons have a number of different receptor types, each mediating a different type of postsynaptic response. Thus, the response of a given cell is determined by which and how many of these receptor types are on the membrane of a given follower cell, and, because of such a differential distribution of receptor types on different follower cells, a single presynaptic neuron can cause excitation in some cells, while eliciting inhibition or complex responses in others.

Although other transmitter candidates [e.g. GABA, glutamate, histamine, glycine (see 1 and 77 for documentation), as well as peptides (e.g. 90) and other neuronal extracts] have been demonstrated to have receptors on molluscan neurons, it is not yet clear whether these receptors serve a synaptic function. Even in the cases where these same substances have been detected in differential quantities in different neurons (see Table 1), their transmitter function in such neurons remains to be established.

MOLLUSCAN MUSCLE

The most conclusive recent evidence concerning the identification of transmitter substances at molluscan neuromuscular junctions has been obtained in *Aplysia* heart, vessels, and gills. Although 5-HT and ACh have been assumed for some time to be the excitatory and inhibitory transmitters, respectively, in molluscan hearts (see 91), the recent coupling in *Aplysia* of biochemical and electrophysiological analyses of identified neurons shown to control monosynaptically heart excitation and inhibition, respectively, has provided a very nice confirmation of conclusions drawn from preparations that have not yet lent themselves to such complete analyses.

Role of Serotonin in Heart Excitation

A motor neuron shown biochemially to be serotoninergic (see RB_{HE}, Table 1) was shown (45) to have direct, monosynaptic excitatory effects on the heartbeat and on blood pressure; these effects could be imitated by exogenously applied serotonin. Dopamine, the only agent with a similar excitatory effect, was 45 times less effective. Finally, the synaptically activated acceleration in heartbeat, as well as the synaptically activated increase in blood pressure, could be blocked by the serotonin antagonist, cinanserin.

Role of ACh in Heart Inhibition

The neurons shown electrophysiologically to inhibit heartbeat by presumed monosynaptic connections were demonstrated biochemically to be cholinergic (see Table 1, LD_{HI}) (45). Exogenous ACh imitated the action of heart-inhibitory neurons, both in producing irregularities in heartbeat and in lowering blood pressure. A similar effect was obtained with the cholinomimetic arecoline. The heart-inhibitory action, like that of exogenous ACh, could be blocked by TEA. The imitation of the heart inhibition by arecoline and its block by TEA suggest that the cholinergic receptor mediating this inhibition is the same as that mediating the K-dependent cholinergic inhibition in molluscan ganglia (see above: also 77, 80).

Role of ACh in Vasoconstriction

The transmitter shown to act on the aortic sphincter musculature was shown also to be ACh (45). The vasoconstrictor neurons of *Aplysia* were shown biochemically to be cholinergic (see Table 1, LB_{VC}). The contractions caused by activation of these neurons could be imitated by ACh, and both the synaptically activated constriction and that caused by exogenous ACh could be blocked by hexamethonium and curare. From the available information, one would anticipate that this cholinergic receptor is the same as that mediating Na-dependent cholinergic excitation in the ganglion. In view of that similarity it could be expected that this response (in spite of being a cholinergic excitatory neuromuscular response) would most probably be unaffected by α-bungarotoxin (78).

Two Excitatory Transmitters Acting on the Gill Muscle

In a recent analysis of the innervation of *Aplysia* gill muscle (39), three major motor neurons were shown to innervate, monosynaptically, different combinations of the main muscle groups. Two of the motor neurons were shown biochemically to be cholinergic (see LDG, Table 1), and the excitatory junctional potentials (e.j.p.'s) resulting from their activation were blocked by hexamethonium. On the other hand, the third motor neuron studied (see L7, Table 1) produces contractions that are unaffected by hexamethonium. Furthermore, the effect produced by this motor neuron (antiflaring of the two halves of the gill) is opposite to that produced by either of the two cholinergic neurons or by exogenous ACh. A biochemical analysis confirmed the conclusions from electrophysiological findings, showing that the neuron L7, unlike the other two motor neurons, is unable to convert choline into

ACh, and does not contain choline acetyltransferase. The experiments thus show that motor neurons liberating different transmitters make monosynaptic contacts with the same muscle fibers, and both elicit e.j.p.'s in those target organs. Thus, excitation appears to be mediated in the same fiber by two different excitatory transmitters.

ARTHROPOD MUSCLE

Recent Investigations on the Role of Glutamate at Insect and Crustacean Muscle

Although much work has been done in the last few years to further characterize the glutamate receptors found on many arthropod muscles, few new data have been obtained that strengthen the position that glutamate is the transmitter at any of these junctions. However, except for the special cases treated in the last sections of this review, glutamate remains the most promising candidate for the transmitter at most of the excitatory junctions on these muscles.

EXTRAJUNCTIONAL RECEPTORS MEDIATING AN INCREASE IN Cl PERMEABILITY Recent investigations on the action of glutamate and other agonists have shown that there are probably at least two different types of glutamate receptors on the insect striated muscle. Cull-Candy & Usherwood (92, 93) showed that whereas glutamate applied iontophoretically at the junctional membrane imitated the depolarizing action of the junctional transmitter (presumably glutamate) glutamate applied to the extrajunctional membrane induced either a biphasic response or a pure hyperpolarizing response. The hyperpolarization caused by glutamate was shown to be due to an increase in membrane permeability to Cl ions. Lea & Usherwood (94, 95) had previously shown that, on the same muscle, ibotenic acid selectively elicited an extrajunctional, hyperpolarizing, Cl-dependent response, and had no effect on the junctional membrane. Cross desensitization was demonstrated between ibotenic acid and glutamate, which strongly suggests that the compounds act on the same extrajunctional receptors. When these extrajunctional receptors were blocked, either by a prior desensitization by ibotenic acid, or by the addition of picrotoxin to the bath, the response of the muscle to bath-applied glutamate was enhanced. These conditions did not enhance the amplitude of the iontophoretically applied junctional response to glutamate, suggesting that the shunting effect of Cl conductance on the depolarization by bath-applied glutamate does not interfere with the localized junctional response (since, as has been shown (96), there is no effect on this response of changes in Cl concentration).

The failure to observe cross desensitization between GABA (the inhibitory transmitter at these junctions which, like ibotenic acid, causes an increase in Cl permeability) and ibotenic acid suggests that the ibotenic acid–glutamate extrajunctional receptor is not the same as that acted upon by the inhibitory transmitter. This is further supported by the finding that ibotenic acid causes a hyperpolarization of all fiber types, even of those which do not receive inhibitory innervation and are not responsive to GABA (95).

In the *striated muscle of the crayfish vas deferens* Florey & Murdock (97) obtained evidence for a similar two-receptor glutamate system. They observed that (*a*) contractures produced by L-glutamate were eliminated in Na-free solutions and were enhanced when external Cl was reduced; (*b*) a glutamate contracture could still be obtained in Na-free solutions if external Cl was also reduced; (*c*) picrotoxin enhanced the glutamate-induced contracture in normal sodium solutions, but eliminated it when Cl was low.

These authors concluded that glutamate increases membrane permeability to both Na and Cl ions and that the glutamate-induced Cl permeability change is the result of an interaction of glutamate with the GABA receptor, since increases in Cl permeability, whether activated by glutamate or GABA, are blocked by picrotoxin. However, in view of the data obtained on locust muscle (see above) it appears probable that in the crayfish vas deferens, as in locust muscle, the glutamate-induced Cl permeability change is mediated by extrajunctional glutamate receptors which, though blocked by picrotoxin, are insensitive to GABA. Experiments testing the effects of ibotenic acid (as well as experiments evaluating cross-desensitization between glutamate and ibotenic acid) would be useful for clarifying whether the Na-independent glutamate effect on the vas deferens is mediated by a glutamate-specific (i.e. GABA insensitive) or a nonspecific (i.e. GABA sensitive) receptor.

EFFECTS OF KAINIC ACID: RELATIONSHIP TO EXTRAJUNCTIONAL RECEPTORS Shinozaki & Shibuya (98) first observed that the depolarization brought about in crayfish muscle by bath-applied L-glutamate was markedly enhanced by adding kainic acid to the bathing medium. In contrast, this compound did not affect the e.j.p.'s which are presumably due to synaptically released glutamate. Takeuchi & Onodera (99), using iontophoretically applied glutamate, have shown that when the application of glutamate is restricted to the junctional membrane, kainic acid causes no enhancement of the response. However, when the application is made over a wider surface, thereby including nonjunctional membrane, kainic acid increases both the amplitude and the duration of the response.

Both groups of authors assume that kainic acid interacts with an extrajunctional glutamate receptor which is assumed to have different pharmacological properties (hence its sensitivity to kainic acid) than does the junctional receptor. That extrajunctional excitatory receptors may exist in arthropod muscle is suggested by the biphasic responses seen in locust extrajunctional membrane (92) and by the spread of glutamate sensitivity seen in denervated locust muscle (100). However, similar efforts to demonstrate an increase in extrajunctional receptors in crayfish muscle (proximal accessory flexor) by denervation have thus far been unsuccessful (101).

FURTHER ANALYSES OF THE IONIC MECHANISMS OF THE JUNCTIONAL GLUTAMATE RESPONSES A number of investigators have continued and refined the analyses of the ionic mechanisms underlying the junctional responses to synaptic activation and to iontophoretically applied glutamate (e.g. 102–104). Their work has revealed that a wide variation exists in the estimated and observed inversion poten-

tial values obtained for these responses, even when studied by the same author in the same preparation. In some cases, the variations were shown to reflect differential contributions of potassium ions to what was otherwise a primarily Na-dependent potential (104). In other instances, it has been suggested that calcium currents make a contribution to the junctional potential (102), accounting presumably for some atypically positive inversion potentials. This conclusion, however, was not reached by other investigators (103). Little seems to be understood as yet about the factors controlling the differential permeabilities of different junctional membranes or of the same type of junctional membrane in different experiments.

FURTHER ANALYSES OF DRUG-RECEPTOR INTERACTIONS BY GLUTAMATE AT THE JUNCTIONAL MEMBRANE Measuring with voltage clamp techniques the junctional currents elicited by application of glutamate with or without leakage from the micropipettes, Dudel (105, 106) demonstrated that when leakage occurs, the dose-response curve yields a log-log slope of 2. In contrast, when no leakage is permitted (using very high resistance pipettes) the resulting dose-response curve yields a log-log slope of 4–6. Furthermore, when the dose-response curve obtained with the no-leakage pipette is used in conjunction with the observed junctional currents to predict the concentration of glutamate reaching the membrane, this concentration is found to be identical with that predicted by the diffusion equation. The steep-sloped S shape of the dose-response curve might avoid interference from the circulating low doses of glutamate found in this preparation, while rendering more effective the high doses liberated synaptically.

Role of ACh in Arthropod Muscle

Until 1972, all studies performed on crayfish or lobster neuromuscular junctions led to the conclusion that glutamate was the best candidate for a mediator at excitatory junctions. The majority of studies yielding this conclusion were performed on the muscles of the walking legs and the claw. These data remain free from contradiction, although it is perhaps worthwhile to note that another amino acid (quisqualic acid) has recently been shown (107) to be much more effective than glutamic acid on the abductor muscle in the first walking leg of the crayfish.

Although the transmitter role of glutamate is not yet under serious reconsideration, there are clear indications that glutamate is not the only excitatory neuromuscular transmitter in these organisms. Futamachi (108) was the first to obtain evidence that in the slow flexor of the crayfish abdomen ACh might serve this function. He demonstrated that the nerve terminal regions innervated by the motor neuron with the largest extracellularly recorded spike were sensitive to iontophoretically applied ACh. The localization of the sensitive spots was highly correlated with the region of synaptic terminals. Furthermore, the e.j.p.'s of this motor neuron were antagonized by curare. These electrophysiological data have received support recently from light and electron microscopy studies (109) showing that the slow abdominal flexor muscle disclosed staining for acetylcholinesterase, whereas the claw abductor muscle (that was used in many studies of glutamate sensitivity) did not. Electron microscopic examination revealed that the AChE staining was confined to the postjunctional membrane.

Both Evoy & Beranek (110) and Lowagie & Gerschenfeld (111) demonstrated that muscle fibers in the slow flexor system gave localized responses to glutamate. These findings are not necessarily inconsistent with the failure of Futamachi to observe glutamate responses under the same conditions he used for observing ACh responses, because he restricted his analysis to the postjunctional membrane innervated by only one of the five excitatory motor neurons innervating this muscle. Some of these muscle fibers may be similar to those of the *Aplysia* gill muscles, which receive excitatory innervation from two neurons liberating different transmitters (39).

The argument for ACh involvement in excitation of crustacean muscle has received more support from the recent work of Marder (64) in the striated musculature of the lobster stomach, innervated exclusively by neurons of the stomatogastric ganglion. Certain neurons (see Table 1) shown to contain choline acetyltransferase innervate the dorsal dilator muscles that respond to ACh but not to glutamate, and elicit e.j.p.'s that can be blocked by curare (64) and potentiated by edrophonium (65). It was also shown (65) that exogeneously applied ACh blocked the e.j.p.'s in this muscle, and that the reversal potentials estimated by extrapolation for the ionophoretic ACh and junctional responses were the same. In contrast, motor neurons in which no choline acetyltransferase could be detected were shown to innervate muscles that do not respond to ACh. Thus, at least two transmitters are being used by the stomatogastric ganglion motor neurons: one, presumably ACh; the other, presumably glutamate.

Octopamine in Arthropods

Much interesting information about the role of octopamine in arthropod nervous systems is now becoming available. Barker et al (28) first reported a high concentration of octopamine in extracts of *Homarus* thoracic connectives, one root of which was shown by Wallace et al (66) to contain small neurons capable of synthesizing and accumulating octopamine. Evans et al (67) showed that these neurons contained large dense-cored vesicles (67), but were unable to locate any conventional synaptic endings made by these neurons. These authors were able to demonstrate release of octopamine from this piece of tissue by depolarization with high K concentrations, and were also able to produce a long-lasting contracture in the dactyl opener muscle by application of 10^{-5}M octopamine.

Barker & Hooper (73) demonstrated octopamine synthesis in parts of the *Panulirus* foregut nervous system which also contain dense-cored vesicles that are likewise uncorrelated with any synaptic terminals (74). Hoyle (60) showed that low (2.5×10^{-9}M) octopamine concentrations mimicked the effects of the dorsal unimpaired medial (DUMETI) neurons of the locust and grasshopper that contain dense-cored vesicles. These neurons were proposed to have a neurosecretory function (60), and were shown (59) to be capable of synthesizing octopamine. Nathanson & Greengard (75) showed an octopamine-sensitive adenyl cyclase in cockroach nervous tissue, and Sullivan & Barker (76) report that concentrations of octopamine that cause physiological changes in lobster and crab ganglia also affect cAMP levels. All of these data are consistent with a neuromodulator role for octopamine in these systems.

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