Multiple Serotonergic Mechanisms Contributing to Sensitization in *Aplysia*: Evidence of Diverse Serotonin Receptor Subtypes

Demian Barbas,[1] Luc DesGroseillers,[1,2] Vincent F. Castellucci,[2,3] Thomas J. Carew,[4,5] and Stéphane Marinesco[4]

[1]*Département de biochimie,* [2]*Centre de Recherche en Sciences Neurologiques, and* [3]*Département de physiologie, Université de Montréal, Québec H3C 3J7, Canada;* [4]*Department of Neurobiology and Behavior, CNLM, University of California–Irvine, Irvine, California 92697, USA*

The neurotransmitter serotonin (5-HT) plays an important role in memory encoding in *Aplysia*. Early evidence showed that during sensitization, 5-HT activates a cyclic AMP-protein kinase A (cAMP-PKA)-dependent pathway within specific sensory neurons (SNs), which increases their excitability and facilitates synaptic transmission onto their follower motor neurons (MNs). However, recent data suggest that serotonergic modulation during sensitization is more complex and diverse. The neuronal circuits mediating defensive reflexes contain a number of interneurons that respond to 5-HT in ways opposite to those of the SNs, showing a decrease in excitability and/or synaptic depression. Moreover, in addition to acting through a cAMP-PKA pathway within SNs, 5-HT is also capable of activating a variety of other protein kinases such as protein kinase C, extracellular signal-regulated kinases, and tyrosine kinases. This diversity of 5-HT responses during sensitization suggests the presence of multiple 5-HT receptor subtypes within the *Aplysia* central nervous system. Four 5-HT receptors have been cloned and characterized to date. Although several others probably remain to be characterized in molecular terms, especially the Gs-coupled 5-HT receptor capable of activating cAMP-PKA pathways, the multiplicity of serotonergic mechanisms recruited into action during learning in *Aplysia* can now be addressed from a molecular point of view.

The marine mollusk *Aplysia* has proven to be a powerful model system for the study of learning and memory. This animal displays several simple forms of nonassociative learning, such as habituation, dishabituation, and sensitization (Pinsker et al. 1970, 1973; Carew et al. 1971), but also more complex forms of associative learning such as classical, operant, and fear conditioning (Carew et al. 1981, 1983; Walters et al. 1981; Lechner et al. 2000a,b; Brembs et al. 2002). The strength of this model system arises from the relative simplicity of its central nervous system (CNS), which contains a small number of neurons, some of which are well characterized both morphologically and electrophysiologically. Thus, one can study a specific synaptic connection in different animals subjected to a wide variety of behavioral training protocols. For this reason, it has been possible to discover many of the mechanisms of learning and memory in this animal at the behavioral, cellular, and molecular levels, and provide direct evidence that certain forms of learning rely on the plasticity of individual synaptic connections (for review, see Kandel 2001).

One of the best characterized forms of learning in *Aplysia* is sensitization, in which a noxious stimulus facilitates an animal's pre-existing response to the presentation of another innocuous stimulus. It has been most thoroughly studied in the defensive reflex responses of *Aplysia*. For example, a mild tactile stimulus applied to the tail evokes the retraction of respiratory organs (gill and siphon) inside the mantle cavity situated on the back of the animal. The strength and duration of this reflex can be enhanced by a noxious electrical stimulation applied to the tail, the head, or the body wall. Memory for this form of sensitization relies at least in part on the strengthening of sensory neuron (SN) to motor neuron (MN) transmission, namely, increase in SN excitability and facilitation of SN–MN synapses. A large amount of experimental data suggests that SN–MN synaptic plasticity depends on the release of the neurotransmitter 5-HT within the *Aplysia* CNS during sensitization training (Brunelli et al. 1976; Mackey et al. 1989; Marinesco and Carew 2002). It is believed that 5-HT activates cyclic adenosine 3′:5′-monophosphate-protein kinase A (cAMP-PKA)-dependent biochemical cascades within SNs, leading to enhanced SN–MN transmission (Brunelli et al. 1976; Castellucci et al. 1980, 1982; Bernier et al. 1982; Abrams et al. 1984; Ocorr and Byrne 1985; Ocorr et al. 1986; Ghirardi et al. 1992; Klein 1993). However, it has become clear that memory processes in *Aplysia* involve more than the activation of cAMP-PKA pathways within SNs. In particular, it has been demonstrated that in addition to modulating SN–MN transmission, 5-HT also acts on several other neuronal cell types within the defensive reflex circuits, and that these actions may involve multiple 5-HT receptor subtypes. In this review, we first summarize the experimental evidence supporting the existence of several serotonergic processes during sensitization. We then review the available data on the different 5-HT receptor subtypes expressed in *Aplysia* tissues and discuss their possible involvement in learning and memory.

The Role of 5-HT in Memory and Synaptic Facilitation in *Aplysia*

The role of 5-HT in sensitization was first suggested in a series of papers by Brunelli et al. (1976) and Castellucci and Kandel (1976), who showed that sensitization of the gill-withdrawal reflex was correlated with an increase in the amount of transmitter

[5]Corresponding author.
E-MAIL tcarew@uci.edu; FAX (949) 824-5161.
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released by sensory neurons (SNs) at their synapses with motor neurons (MNs), a phenomenon termed presynaptic facilitation (Castellucci and Kandel 1976). This phenomenon could be mimicked by exogenous application of 5-HT or intracellular injection of cAMP, and blocked by the 5-HT antagonist cinanserin (5-HT_2 antagonist; Brunelli et al. 1976), and was later shown to be blocked by cyproheptadine (5-HT_{1-2} antagonist; Mercer et al. 1991). Although the specificity of these drugs for *Aplysia* 5-HT receptors has not been fully characterized, these data suggest that 5-HT is responsible for the presynaptic facilitation observed during sensitization.

Considerable experimental work was then devoted to identifying the serotonergic neurons responsible for presynaptic facilitation, and determining whether 5-HT was indeed released in the *Aplysia* CNS during sensitization training. Mackey et al. (1989) showed that a pair of symmetrical 5-HT neurons in the cerebral ganglion, the CC3 (CB1) cells (see Xin et al. 2001 for change in nomenclature) were activated by noxious electrical stimulation of the tail. Moreover, activation of one CC3 (CB1) cell by intracellular current injection was sufficient to produce facilitation of SN–MN synapses as well as spike broadening in SNs in the abdominal ganglion (Mackey et al. 1989; see also Wright et al. 1995). However, as Mackey and colleagues point out, CC3 (CB1) modulation of SN–MN transmission was modest and short-lived compared to modulation by exogenously applied 5-HT. Moreover, it is not known whether the effects of CC3 (CB1) stimulation can be blocked by specific 5-HT receptor antagonists. Thus, the precise role of CC3 (CB1) during sensitization and the respective effects exerted by 5-HT or by possible cotransmitters that could be released by this neuron remain to be determined. Using direct electrochemical methods, Marinesco and Carew (2002) were able to detect a transient release of 5-HT in the *Aplysia* CNS in response to electrical stimulation of the tail-nerve P9, an analog of the noxious stimulation of the tail used to produce sensitization. This release lasted about 30–40 sec and peaked around 100–200 nM in the neuropil surrounding tail SN–MN synapses or SN cell bodies (Marinesco and Carew 2002). Increased 5-HT levels in the hemolymph have also been detected during long-term sensitization (Levenson et al. 1999).

For many years, the receptors mediating the actions of 5-HT during learning remained unknown. The lack of information on the 5-HT receptors expressed in the *Aplysia* CNS has made it difficult to investigate the role of 5-HT in memory at the behavioral level. Indeed, few pharmacological tools are available for accurate manipulation of 5-HT pathways in freely moving animals. The first and only behavioral study to date was performed by Glanzman et al. (1989). These authors chemically lesioned the serotonergic system in *Aplysia* using high doses of the neuronal toxin 5,7-dihydroxytryptamine (5,7-DHT). They point out that lesioned animals showed major behavioral impairment such as inability to produce locomotion or attach to a substrate. The animals nonetheless displayed defensive reflexes susceptible to habituation. Glanzman and colleagues (1989) observed that dishabituation and synaptic facilitation were greatly decreased in lesioned animals, suggesting that 5-HT release during training is necessary for this form of memory. The effects of this drug on sensitization were not determined (Glanzman et al. 1989). As we will discuss below, designing ways to interfere with specific *Aplysia* 5-HT receptor subtypes in freely moving animals would allow confirmation and further exploration of this important finding.

Modulation of Defensive Reflex Circuits by 5-HT During Sensitization

The *Aplysia* CNS, with its relatively small number of neurons, is a useful model system for mapping neuronal circuits controlling specific aspects of behavior. In this regard, the neuronal circuits mediating defensive reflexes have been extensively studied. Specifically, these include the tail-induced and siphon-induced tail/siphon withdrawal reflexes (Fig. 1). These reflexes involve three central ganglia: the pleural, pedal, and abdominal ganglia. A tactile stimulus applied to the tail or siphon activates tail or siphon SNs, which excite MNs through monosynaptic (SN–MN synapses) and polysynaptic pathways (for a more detailed review, see Cleary et al. 1995; Frost and Kandel 1995). More than 10 types of interneurons are known to participate in these polysynaptic pathways between SNs and MNs: (1) L25, R25, L26, and L33 are recruited during siphon withdrawal reflex and respiratory pumping (Kanz et al. 1979; Byrne 1983; Eberly and Pinsker 1984; Frost and Kandel 1995), (2) L16, L29, L30, L33, and L34 mediate siphon withdrawal reflex only, and (3) Pl 4 and Pl 17 are involved in reflex tail withdrawal (Buonomano et al. 1992; Cleary and Byrne 1993; Xu et al. 1994). Moreover, Pl 17 is viewed as possibly providing a linkage between pleural and abdominal reflex circuits (Cleary and Byrne 1993). Four types of interneurons are excitatory (L25, R25, L29, and L34), five are inhibitory (L16, L26, L30, L33, and Pl 4), and Pl 17 seems to be excitatory to tail MNs and inhibitory to tail SNs (Fig. 1; Cleary et al. 1995). When excitatory and inhibitory interneurons are recruited during siphon reflex retraction, their synaptic potentials sum at the level of MNs and various interneurons. Although inhibitory interneurons (L16, L30, Pl 4; see Fig. 1) are present within the circuit, activation of this polysynaptic pathway usually results in a stronger and longer excitation of siphon MNs. Therefore, the role of polysynaptic pathways is usually viewed as amplifying and prolonging the monosynaptic excitation coming from SNs (Trudeau and Castellucci 1992; White et al. 1993; Antonov et al. 1999).

Many neurons within this circuit are modulated by both sensitization training and by 5-HT application. Interestingly, 5-HT release in response to tail-nerve shock has been directly demonstrated in the pleural, pedal, and abdominal ganglia, which are the sites of the neuronal circuits for reflex tail or siphon withdrawal (Marinesco and Carew 2002; Bristol et al. 2003). 5-HT is therefore in a position to modulate virtually all of the neuronal elements involved in this circuit during sensitization training.

Fitzgerald and Carew (1991) and Trudeau and Castellucci (1992, 1993) sought to distinguish the modulation of mono- and polysynaptic pathways during sensitization. They determined that, whereas monosynaptic SN–MN connections were facilitated by 5-HT, polysynaptic pathways were usually inhibited (Fitzgerald and Carew 1991; Trudeau and Castellucci 1992, 1993). At least four sets of synapses have been shown to be inhibited by 5-HT (Fig. 1): L16 → LE (Storozhuk and Castellucci 1999a), L29 → LFS (Frost et al. 1988; Bristol et al. 2001), L30 → L29 (Frost et al. 1988; Fisher and Carew 1993), and Pl 4 → tail SNs *and* MNs (Xu et al. 1995). Some of these synapses (L29 → LFS, L30 → L29) can undergo the same type of modulation during sensitization training (Frost et al. 1988; Fischer and Carew 1993; Bristol et al. 2001). Moreover, 5-HT decreases the excitability of Pl 4, whereas it usually enhances excitability in tail and siphon SNs. Finally, the siphon is innervated by at least four populations of SNs (LE, rLE, RE, and RF; Frost and Kandel 1995), that can be differentially modulated by 5-HT. For example, 5-HT increases LEs' excitability and facilitates their synapses onto follower neurons, whereas it depresses RF synapses (Fig. 1; Storozhuk and Castellucci 1999b).

5-HT modulation in interneurons is not limited to synaptic plasticity or excitability changes. The synapses from the inhibitory L30 interneurons onto L29 excitatory interneurons provide an example of complex modulation that is mediated both by 5-HT and by sensitization training. These inhibitory synapses undergo a form of activity-dependent short-term synaptic en-

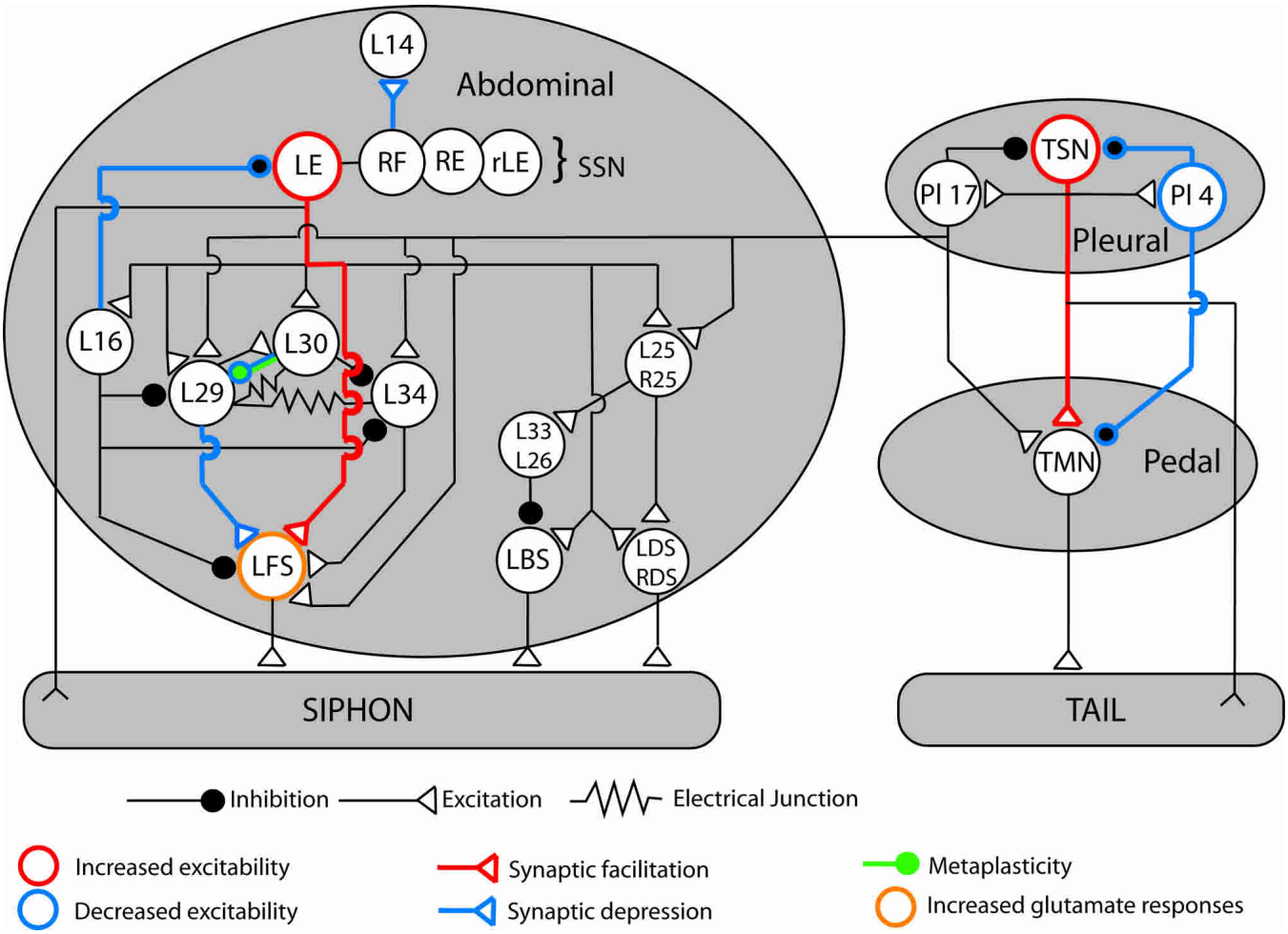

Figure 1 Schematic diagram of the neuronal circuit mediating tail and siphon withdrawal reflex. Neuronal cell bodies are located in three central ganglia (pleural, pedal, and abdominal). A tactile stimulus applied to the tail or siphon activates tail (TSN) or siphon sensory neurons (SSN), which excite MNs through monosynaptic (SN–MN synapses) and polysynaptic pathways depending on interneurons. 5-HT exerts multiple actions on the circuit: It usually induces synaptic depression and/or decreases in excitability in interneurons (blue) and synaptic facilitation and increase in excitability in SNs (red). L14, an ink gland MN, illustrates 5-HT-induced synaptic depression in RF SNs. 5-HT also induces metaplasticity at L30-L29 synapses (green) and increases glutamate responses in LFS MNs (orange). Adapted from Cleary et al. 1995.

hancement induced by the activation of the L30 neurons (Fischer and Carew 1993, 1995). For example, when L30 interneurons fire in response to a mild tactile stimulation, synaptic efficacy at the L30–L29 synapses is enhanced, thus contributing to inhibition of the siphon-withdrawal reflex circuit (Fischer and Carew 1995; Fischer et al. 2000). Both 5-HT and sensitization training block the expression of this form of synaptic plasticity, thereby reducing the potential for inhibition within the circuit. This higher-order form of plasticity reflects an example of "metaplasticity" (Fischer et al. 1997).

In addition to SN and interneuron modulation, a direct action of 5-HT on MNs has also been described. It involves facilitation of AMPA-type responses to glutamate, the putative neurotransmitter released by SNs, in cultured LFS neurons (Chitwood et al. 2001), and could possibly take place during sensitization training.

We should note that the neuronal circuits mediating defensive reflexes can also be modulated through a variety of nonserotonergic mechanisms. For example, small cardioactive peptide B (SCP_B) facilitates SN–MN synapses (Abrams et al. 1984; Pieroni and Byrne 1992) and L16 → LE connections (Storozhuk and Castellucci 1999a), and has excitatory effects on Pl 4 (Xu et al. 1995). Phe-Met-Arg-Phe-amide (FMRFamide) has been shown to inhibit SN–MN transmission (Mackey et al. 1987; Dale and Kandel 1990; Critz et al. 1991; Pieroni and Byrne 1992; Sun et al. 1996), increase the SN firing threshold (Billy and Walters 1989), and modulate specific postsynaptic MNs involved in defensive reflexes (Belkin and Abrams 1993). However, it is not known whether these peptides are released in the CNS during sensitization training. Facilitation of SN–MN transmission can also be induced by intracellular stimulation of interneurons L29 and L28 (Hawkins 1981a,b). These interneurons act directly on siphon SNs using an unknown transmitter; it has been determined that the transmitter is not 5-HT (Kistler Jr. et al. 1985; Hawkins and Schacher 1989). Finally, an increasing amount of evidence indicates that, in addition to heterosynaptic processes involving the release of modulatory transmitters onto the neuronal circuit, homosynaptic activity-dependent plasticity also occurs during learning, namely posttetanic potentiation (Walters and Byrne 1984; Frost et al. 1988; Schacher et al. 1990; Bristol et al. 2001) and long-term potentiation (Murphy and Glanzman 1996, 1997; Antonov et al. 2001, 2003). These types of synaptic plasticity are beyond the scope of this review.

Overall, these data indicate that modulation of defensive

reflex circuits by 5-HT involves a variety of different cell types that respond differently to 5-HT. As we will discuss below, it is likely that, when released in the *Aplysia* CNS during sensitization training, 5-HT acts on several subtypes of receptors that are differentially expressed by SNs, interneurons, and MNs and modulate synaptic transmission and excitability in different ways.

Biochemical Pathways Activated by 5-HT in SNs

Although the neuronal circuit mediating siphon reflex retraction involves many different cell types, most of which can be modulated by 5-HT, most experimental studies on synaptic plasticity in *Aplysia* have focused on SNs and SN–MN synapses. Indeed, the SN–MN synapses have provided a valuable locus where the cellular and molecular mechanisms underlying memory have been studied. SN–MN synapses are activated during tail or siphon reflex retraction, and their plasticity contributes significantly to simple forms of memory such as sensitization, dishabituation, and classical conditioning (Trudeau and Castellucci 1992; Antonov et al. 1999, 2001, 2003). For example, LE → LFS synapses account for about 30% of the siphon-induced siphon withdrawal reflex efficacy (Antonov et al. 1999). Moreover, behavioral sensitization and SN–MN facilitation display similar temporal profiles and molecular mechanisms. For example, a single noxious stimulus usually gives rise to short-term sensitization lasting 20–30 min, whereas four or five spaced shocks induce intermediate-term and long-term sensitization lasting 90 min and several days, respectively (Pinsker et al. 1973; Castellucci and Kandel 1976; Sholz and Byrne 1987; Sutton et al. 2001, 2002). Similarly, a single exogenous 5-HT application gives rise to short-term facilitation, whereas five applications produce intermediate-term and long-term facilitation at SN–MN synapses (Frost et al. 1985; Montarolo et al. 1986; Sholz and Byrne 1987; Ghirardi et al. 1995; Mauelshagen et al. 1996; Sutton and Carew 2000). In addition, sensitization and facilitation of SN–MN synapses share common molecular requirements: (1) short-term sensitization and facilitation rely on covalent modification of proteins, (2) intermediate-term, on synthesis of new proteins, and (3) long-term, on RNA and protein synthesis (Castellucci et al. 1986, 1989; Montarolo et al 1986; Ghirardi et al. 1995; Sutton et al. 2001). Finally, a detailed analysis of various neuronal loci during long-term sensitization revealed that, although short-term sensitization is correlated with synaptic plasticity at multiple cell types (SNs, interneurons, etc.), the neuronal correlates of long-term sensitization appear more restricted to SNs (Cleary et al. 1998). For these reasons, most of the studies aimed at understanding the mechanisms of synaptic plasticity in *Aplysia* have used facilitation of SN–MN connections as a model system, leaving 5-HT inhibition in interneurons almost completely unexplored.

Considerable evidence indicates that a single application of 5-HT onto SNs can activate both PKA and protein kinase C (PKC; for review, see Byrne and Kandel 1996). For example, elevation of intracellular cAMP levels and increased PKA activity can be detected following a single pulse of 5-HT (Bernier et al. 1982; Castellucci et al. 1982; Abrams et al. 1984; Ocorr and Byrne 1985; Ocorr et al. 1986; Müller and Carew 1998). Similarly, 5-HT leads to the translocation to the membrane of the Ca^{2+}-dependent isoform of PKC (Saktor and Schwartz 1990; Kruger et al. 1991; Sossin and Schwartz 1992; Braha et al. 1993; Sossin et al. 1993) and an increase in PKC activity (Sossin et al. 1994). Interestingly, recent studies by Manseau et al. (2001) indicated that blocking a Ca^{2+}-independent PKC isoform disrupted facilitation induced by 5-HT at depressed SN–MN synapses, whereas blocking the Ca^{2+}-dependent isoform had no effect. Both PKA and PKC are thought to (1) phosphorylate specific ion channels that increase membrane excitability and induce spike broadening, and (2) modulate vesicle mobilization and exocytosis to increase transmitter release. For example, PKA, by phosphorylating S-type K^+ channels, reduces their open time (Siegelbaum et al. 1982). This effect contributes to the increase in membrane excitability and spike broadening following 5-HT application (Klein et al. 1982; Pollock et al. 1985; Baxter and Byrne 1989). Voltage-dependent K^+ channels (K_v) can also be phosphorylated by both PKA and PKC, and seem to be involved specifically in spike broadening (Baxter and Byrne 1989, 1990; Goldsmith and Abrams 1992; Sugita et al. 1992). Spike broadening is an important mechanism for enhancing synaptic transmission through increased Ca^{2+} influx in nerve terminals. However, 5-HT can increase transmitter release independently of spike broadening, by enhancing the mobilization of transmitter vesicles to the active zone (Klein 1993, 1994). Both PKA and PKC seem capable of producing this effect (Hochner et al. 1986; Braha et al. 1990; Sugita et al. 1992).

Interestingly, PKA and PKC are differentially recruited depending on the duration of 5-HT application and on the state of the synapse. For example, PKA blockers are more effective in preventing synaptic facilitation after short 5-HT applications, whereas PKC blockers are more effective after long exposures (Braha et al. 1990; Hochner and Kandel 1992; Sugita et al. 1992). Similarly, facilitation of depressed synapses is blocked by inhibitors of PKC but not PKA, whereas the converse has been shown for nondepressed synapses (Braha et al. 1990; Goldsmith and Abrams 1991; Ghirardi et al. 1992). This state- and time-dependence of PKA and PKC recruitment by 5-HT (Byrne and Kandel 1996) remains unexplained. It is possible that 5-HT activates two different receptor subtypes coupled to diacylglycerol (DAG) and cAMP, respectively, or that a single receptor subtype could bind to two different G proteins. This question will remain unanswered until 5-HT receptors expressed by SNs have been fully characterized.

In addition to PKA and PKC, repeated pulses of 5-HT, which give rise to long-term facilitation, are able to recruit another set of protein kinases that are not activated by a single application. For example, the mitogen-activated protein kinase (MAPK), also called extracellular-signal-regulated kinase (ERK), is activated in response to repeated pulses of 5-HT and translocates to the nucleus, where it modulates gene expression necessary for long-term facilitation (Martin et al. 1997; Michael et al. 1998; Sharma et al. 2003). Moreover, 5-HT-induced ERK activation and long-term synaptic facilitation both require activation of tyrosine kinases (presumably receptor tyrosine kinases or RTKs; Purcell et al. 2003). What makes repeated 5-HT pulses capable of activating ERK and tyrosine kinases, when a single application is unable to do so? Again, it is likely that a better knowledge of *Aplysia* 5-HT receptors will help elucidate this important question.

Because of their central role in memory processes and synaptic plasticity, considerable effort has been devoted to characterizing *Aplysia* 5-HT receptors, from as early as the 1960s. The most striking progress, however, has been obtained within the last 10 years. During this time, the cloning and characterization of four different 5-HT receptors has been reported. We will now turn our attention to a discussion of the properties of these receptors as well as their potential implication in memory processes.

Evolutionary Divergence of 5-HT Receptors

In general, 5-HT receptors are classified and grouped into seven families on the basis of sequence identity and on the nature of the second-messenger systems to which they are coupled. Six of these families are composed of G-protein-coupled metabotropic receptors (or GPCRs). The 5-HT_1 and 5-HT_5 (Gi-coupled recep-

tors) inhibit adenylyl cyclase, whereas 5-HT_4, 5-HT_6, and 5-HT_7 (Gs-coupled receptors) subtypes activate its activity. Only two subtypes are not linked to the adenylyl cyclase pathway: (1) 5-HT_2 receptors are coupled to phospholipase C (Gq-coupled receptors) and stimulate phosphoinositide metabolism, and (2) 5-HT_3 subtypes are ionotropic receptors and thus are not composed of GPCRs (Hoyer et al. 1994; Peroutka 1995). Previous phylogenetic studies suggested that mammalian 5-HT receptor subtypes emerged from gene duplication, followed by mutations and sequence drift (Vernier et al. 1995; Tierney 2001). Early molecular events first led to the divergence of three major classes of paralogous 5-HT receptors: the 5-HT_1 (5-$HT_{1,5,7}$ receptors), the 5-HT_2, and 5-HT_6 classes which existed about 750 million years ago (Mya). Further division within the 5-HT_1 class occurred 600 Mya, when 5-HT_5 and 5-HT_7 subtypes diverged from the 5-HT_1 subtype. These divergences predate the evolution of vertebrates from invertebrates, which also occurred about 600 Mya. Therefore, members of the three major classes are likely to be found in invertebrate species. However during evolution, subsequent independent differentiation of each subtype within each class of vertebrate and invertebrate receptors makes it difficult to classify invertebrate 5-HT receptors into specific vertebrate subtypes (Peroutka 1994; Peroutka and Howell 1994; Tierney 2001).

Early Characterization of 5-HT Receptors Expressed by *Aplysia* Neurons

5-HT mediates a great variety of functions in *Aplysia* neurons, probably through multiple G-protein coupled receptors. Serotonergic effects were first reported on neurons (Gerschenfeld and Tauc 1961; Cedar and Schwartz 1972), heart (Koester et al. 1973), and gill (Kebabian et al. 1979). In 1974, Gerschenfeld and Paupardin-Tritsch described six different types of responses following application of 5-HT to *Aplysia* neurons: four of the responses (named *A*, *A′*, *B*, and *C*) involved an increase in membrane conductance, whereas the other two (named α and β) involved a decrease in membrane conductance (Gerschenfeld and Paupardin-Tritsch 1974). Pellmar and Carpenter (1980) identified a seventh type of response to 5-HT: a voltage- and calcium-dependent response. These different responses were distinguished by voltage clamp experiments in terms of their time course, ionic selectivity, and whether they were mediated by an increase or decrease in membrane conductance. Although these electrophysiological responses could in principle be mediated by a single 5-HT receptor coupled to different intracellular cascades, these experiments are regarded as the first indications of a variety of potential 5-HT receptor subtypes in the CNS of *Aplysia*.

The presence of multiple 5-HT receptors was confirmed by biochemical techniques. Saitoh and Shih (1987) found that an agonist of 5-HT_{1A} receptors, 1-[2-(4-azidophenyl)ethyl]4-(3-trifluoromethylphenyl)piperazine (azido-PAPP), is a suitable photoaffinity labeling probe for 5-HT receptors in *Aplysia*. They found that five labeled polypeptides were separated by SDS-PAGE upon incubation of [^{3}H]azido-PAPP with total neuronal membranes and UV irradiation. The labeling was abolished by 5-HT, which competes for binding on the same receptors. These results suggested the existence of at least five 5-HT receptor subtypes.

As there is increasing interest in characterizing the role of 5-HT as a neurotransmitter in *Aplysia*, it is important to identify the pharmacological properties of 5-HT receptors in this animal. In vertebrate nervous tissues, receptor binding techniques have proven to be valuable for the identification and pharmacological characterization of 5-HT receptors. In *Aplysia*, Drummond et al. (1980), using [^{3}H]LSD (lysergic acid diethylamide, a nonselective 5-HT receptor agonist) binding assays and 5-HT-induced modulation of adenylyl cyclase activity, reported the first detailed distribution of 5-HT receptors in various neuronal and nonneuronal tissues. Specific 5-HT-sensitive [^{3}H]LSD binding was found in the gill, heart, buccal muscles, and in all ganglia of the CNS of *Aplysia*. After localization of 5-HT receptors, stimulation of adenylyl cyclase by 5-HT in membranes of *Aplysia* ganglia, muscles, and connective nerves was measured, indicating the presence of 5-HT Gs-coupled receptors. Increase in 5-HT-sensitive adenylyl cyclase activity correlated well with the amount of 5-HT-sensitive [^{3}H]LSD binding sites in most tissues. High density of 5-HT receptors in pleuro-abdominal connective nerves and their presence in the connective tissue sheaths surrounding the ganglia suggest that not all 5-HT receptors are located at cell bodies and synapses.

Kadan and Hartig (1988) then took advantage of the improved sensitivity of ^{125}I-isotope labeling to pursue the mapping of 5-HT receptors in *Aplysia*. They found that ^{125}I-LSD labeled a population of high-affinity serotonergic sites in *Aplysia* ganglia. 5-HT receptors were primarily located within the neuropil, although a subset of neuronal somata was also labeled. Those authors next determined the potency of various pharmacological compounds to compete for ^{125}I-LSD binding sites on sections from pedal or abdominal ganglia. The order of potency was: methysergide (5-$HT_{1\text{-}2\text{-}7}$ antagonist) > cyproheptadine (5-$HT_{1\text{-}2}$ antagonist) > mianserin (antagonist/inverse agonist of 5-HT_2 receptors) > cinanserin (effective antagonist of 5-HT responses in *Aplysia*; Brunelli et al. 1976; Newlin et al. 1980) > 5-HT > ketanserin (5-HT_2 antagonist) > bufotenine (5-$HT_{1\text{-}2}$ agonist) > 8-OH-DPAT (5-HT_{1A} agonist). Kadan and Hartig (1988) observed a decrease in the affinity of 5-HT for ^{125}I-LSD binding sites in the presence of Gpp(NH)p (5′-guanylylimidodiphosphate), a poorly metabolized analog of guanine nucleotide triphosphate (GTP), suggesting a coupling of 5-HT receptors to G-proteins in *Aplysia* CNS. These data indicated that ^{125}I-LSD binding sites in the *Aplysia* nervous system are regionally distributed, exhibit specific pharmacological binding properties, and are coupled to G-proteins. This is consistent with labeling of functional heterogeneous 5-HT receptors. By comparing the pharmacological properties of the ^{125}I-LSD labeled sites with the properties of the six distinct serotonergic receptor types identified by Gerschenfeld and Paupardin-Tritsch (1974), Kadan and Hartig (1988) suggested that LSD antagonizes the neuronal responses mediated by three of the six electrophysiologically characterized 5-HT receptors (the A, B, and C types). However, the pharmacological profiles for each of these three types differ to some degree from the profile they observed for ^{125}I-LSD binding. For example, compounds used by Gerschenfeld and Paupardin-Tritsch (1974) to inhibit the A and C responses did not block ^{125}I-LSD binding, even at 10 times the concentrations previously used by Gerschenfeld and Paupardin-Tritsch (1974).

Evans et al. (1991), also using ^{125}I-LSD, provided further characterization of the localization of 5-HT receptors, focusing on the five major neurons which constitute the abdominal ganglion left upper quadrant (L1, L2, L3, L4, and L6). They also investigated in more detail the electrophysiological properties of the responses they produced following activation by 5-HT. First, intense labeling was only observed on the soma of a symmetrically located pair of cells in the abdominal ganglion of *Aplysia californica*: L1 and R1. This binding was blocked by micromolar concentrations of 5-HT and lower concentrations of the serotonergic antagonists cyproheptadine and mianserin, confirming Kadan and Hartig's (1988) results. Second, electrophysiological investigation of 5-HT responses of the neurons in the left upper quadrant revealed a range of 5-HT responses. Cells L3 and L6 showed an increased K^+ conductance in response to 5-HT that is not blocked by cyproheptadine or mianserin. Cells L2 and L4 displayed a biphasic response to 5-HT: an increase in Na^+ con-

ductance, which could be blocked by cyproheptadine or mianserin, followed by a voltage-dependent Ca^{2+} conductance which was blocked by Co^{2+} but not the serotonergic antagonists. Cell L1 and its symmetrical partner, R1, in addition to the Na^+ and Ca^{2+} responses observed in L2 and L4, displayed an increase in Cl^- conductance that can be inhibited by LSD, cyproheptadine, and mianserin.

These early studies were the first attempts to identify the distribution and pharmacological properties of diverse 5-HT receptors in *Aplysia*. They demonstrated the presence of 5-HT receptors in virtually all *Aplysia* tissue types and especially in the CNS. They also suggested the existence of at least six receptor subtypes. The development of molecular biological techniques and the possibility of cloning and expressing individual 5-HT receptor subtypes in cell lines allowed further elucidation of the electrophysiological and pharmacological properties of the serotonergic pathways in *Aplysia*.

Structural and Pharmacological Characterizations and Tissue Distribution of Cloned 5-HT Receptors

Cloning of 5-HT Receptors in Aplysia

Four 5-HT receptors have been characterized in *Aplysia* to date. Li et al. (1995) isolated potential genes that encode *Aplysia* 5-HT receptors by performing a PCR analysis of *Aplysia* genomic DNA. In order to amplify potential 5-HT receptors, they used degenerate PCR primers whose sequences were based on conserved peptide sequences found in the sixth and the seventh transmembrane domains of all known serotonergic GPCRs. Two different genomic clones, *Ap5-HT$_{B1}$* and *Ap5-HT$_{B2}$*, were isolated. The *Ap5-HT$_{B1}$* gene codes for a protein of 453 amino acids (GenBank accession no. L43557) containing seven hydrophobic putative transmembrane domains, a relatively short third intracellular loop, and a long C-terminal tail. Genomic analysis further demonstrates that the gene is intronless. The *Ap5-HT$_{B2}$* gene is also intronless and codes for a closely related protein of 422 aa (GenBank accession no. L43558) which shares 90% amino acid sequence identity with the Ap5-HT$_{B1}$ within the transmembrane domains and adjacent regions. The N-terminal extracellular domain and the extracellular loops I and II are less conserved. More divergence occurs within the third intracellular loop and the C-terminal tail. It is possible that other related receptors are present in the *Aplysia* genome, because a Southern blot analysis of genomic DNA, probed with the coding region of Ap5-HT$_{B1}$, revealed five distinct bands. Dendrogram analysis of the amino acid sequences of cloned 5-HT receptors indicated that Ap5-HT$_{B1}$ and Ap5-HT$_{B2}$ are only distantly related to the rest of the known 5-HT receptors (Fig. 2). They could not be readily grouped within any of the mammalian subgroups based on amino acid sequence identity.

A third 5-HT receptor gene was cloned and characterized from *Aplysia* and named *5-HT$_{ap1}$* (Angers et al. 1998). Degenerate primers and PCR amplification were used to screen CNS and kidney cDNA libraries. A cDNA coding for a putative protein of 492 aa containing seven stretches of hydrophobic residues, a relatively large third cytoplasmic loop, and a short C-terminal tail, features common to several 5-HT$_1$ receptors, was isolated (GenBank accession no. AF041039; Boess and Martin 1994; Gerhardt and van Heerikhuizen 1997). Southern blot analysis of genomic DNA revealed that the *5-HT$_{ap1}$* gene is probably intronless and does not have a close homolog in the *Aplysia* genome. Dendrogram analysis of amino acid sequence within the transmembrane domains also suggests that *5-HT$_{ap1}$* is associated with the mammalian 5-HT$_1$ receptor family (Fig. 2).

The same cloning strategy was used to obtain the sequence

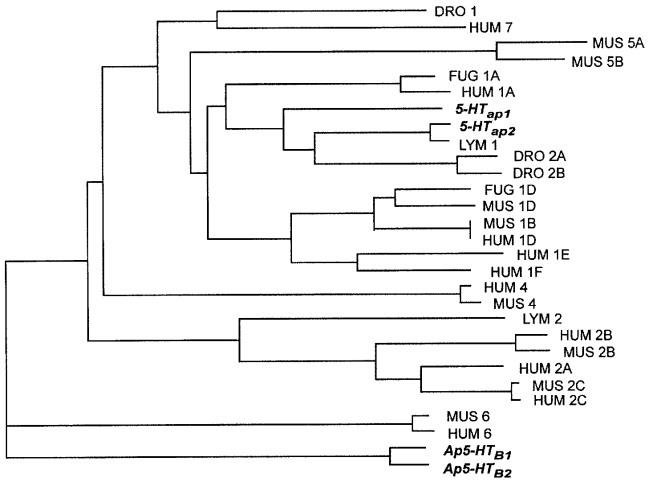

Figure 2 Dendrogram analysis of different members of the 5-HT receptor superfamily. Sequences that were used for phylogenetic analysis were retrieved from the GenBank database. The sequences of serotonin receptors were compared and aligned using ClustalW (Thompson et al. 1994), which was executed from GDE (Genetic Data Environment; J. Felsenstein 1993, PHYLIP, Phylogeny Inference Package, version 3.5.1c and 3.6, University of Washington, Seattle, WA). Only amino acid positions that could be aligned without ambiguity were used for the analysis. The alignment was then used for phylogenetic comparisons using the PHYLIP package. Analysis was performed with a bootstrap procedure that computes the probability of occurrence of the branches for 1000 possible trees. Branching order was determined using the Fitch-Margoliash algorithm included in the PHYLIP package. Only branches occurring in >800 trees are represented. DRO, *Drosophila*; FUG, *Fugu rubripes* (pufferfish); LYM, *Lymnaea*; HUM, human; MUS, mouse.

of a fourth *Aplysia* 5-HT receptor gene, named *5-HT$_{ap2}$* (GenBank accession no. AF372526; Barbas et al. 2002). The cDNA contains an open reading frame encoding a protein of 567 aa with a predicted molecular mass of 63 kD. Hydrophobicity analysis reveals the presence of the seven transmembrane domains characteristic of GPCRs. Its large third cytoplasmic loop, its short C-terminal tail, and the observation that the *5-HT$_{ap2}$* gene is probably intronless and does not have a close homolog in the *Aplysia* genome are reminiscent of members of the vertebrate 5-HT$_1$ receptor family and similar to the 5-HT$_1$-like *Aplysia* receptor 5-HT$_{ap1}$. Amino acid sequence comparisons between the 5-HT$_{ap2}$ receptor and other 5-HT receptors show significant sequence identity to the 5-HT$_1$-like receptors, and a dendrogram analysis places 5-HT$_{ap2}$ within the family of mammalian 5-HT$_1$ receptors (Fig. 2). Sequence analysis also reveals that 5-HT$_{ap2}$ has close amino acid identity (68%) with the *Lymnaea stagnalis* 5-HT receptor 5-HT$_{lym}$ (Sugamori et al. 1993), suggesting that they may be orthologous.

Ap5-HT$_{B1}$ and Ap5-HT$_{B2}$ Receptors Activate Phospholipase C and Have Different Expression Patterns

To identify their pharmacological profiles, Ap5-HT$_{B1}$ and Ap5-HT$_{B2}$ receptors were stably expressed in HEK 293 cells (Li et al. 1995). In response to 5-HT, both receptors stimulate phospholipase C activity, reaching a plateau at 100 nM. Therefore, the stimulation of these two receptors might lead to the activation of PKC. The estimated EC_{50} for 5-HT was 1.8 nM for Ap5-HT$_{B1}$ and 1.5 nM for Ap5-HT$_{B2}$. Methiothepin and spiperone, which are respectively nonselective 5-HT and 5-HT$_{2A/1}$ antagonists, both prevent the 5-HT-dependent stimulation of phospholipase C at 10 μM concentration. However, cyproheptadine (10 μM), another 5-HT$_{1\text{-}2}$ antagonist, has no effect. Thus, although Ap5-HT$_{B1}$ and Ap5-HT$_{B2}$ do not appear to be the invertebrate homologs of

the mammalian 5-HT_2 receptors, they do have similar coupling to phospholipase C.

RT-PCR experiments on mRNA isolated from various tissues of *Aplysia* showed that Ap5-HT_{B1} mRNA is expressed in spermatheca and ovotestis, whereas Ap5-HT_{B2} mRNA is detected only in the CNS. As Ap5-HT_{B1} and Ap5-HT_{B2} have similar pharmacological profiles but different expression patterns, Li et al. (1995) suggested that these two receptors might result from the recent duplication of an ancestral gene, which placed the coding sequence of the ancestral gene under the control of totally different promoter sequences.

The 5-HT_{ap1} Receptor Is Negatively Coupled to Adenylyl Cyclase

The pharmacological profile and expression pattern of the 5-HT_{ap1} receptor were also characterized (Angers et al. 1998). This receptor, fused to a c-myc epitope at its N terminus, was expressed in mammalian HEK 293 cells. Isolated membranes bind [^{3}H]LSD in a saturable and dose-dependent manner with an estimated K_d of 0.56 nM. The rank order of potency of various serotonergic agonists and antagonists for inhibition of [^{3}H]LSD binding is given in Table 1. 5-HT_{ap1} shows high-affinity binding to 5-HT which is comparable to that of the mammalian 5-$HT_{1A,B,D,F}$, and 5-HT_7 receptors. From these results, Angers et al. (1998) concluded that the pharmacological profile of 5-HT_{ap1} seems to be related to the mammalian 5-HT_1 and 5-HT_7 receptor profiles. The structure and function of the 5-HT_{ap1} receptor are probably closely related to those of an ancestral receptor, which existed before the divergence of the 5-HT receptor subtypes in vertebrates. It appears to have kept the characteristics of more than one receptor subtype, possibly related to a prototype of the early 5-$HT_{1,7}$ receptors.

As expected from the structure and pharmacological profile of 5-HT_{ap1}, stimulation of the receptor with 5-HT in HEK 293 cells inhibits forskolin-induced cAMP accumulation in a dose-dependent manner (Angers et al. 1998). This inhibition is blocked in the presence of 100 nM of the antagonist methiothepin. These experiments indicate that 5-HT_{ap1} is functionally coupled to the mammalian Gi subunit and inhibits adenylyl cyclase and cAMP accumulation (Angers et al. 1998).

Table 1. Comparison of the Affinities of Various Compounds That Compete With the Binding of [^{3}H]LSD to the Membranes of Cells Transfected With the 5-HT_{ap1} and 5HT_{ap2} cDNA

		K_i (nM)	
Drug	Specificity	5-HT_{ap1}[a]	5-HT_{ap2}[b]
5-CT	5-HT_1 agonist	1.04	162
Methiothepin	Nonselective 5-HT antagonist	2.62	0.3
PAPP	5-HT_{1A} agonist/5-HT_{1D} antagonist	9.50	182
5-HT		13.23	241
Clozapine	5-HT_2 antagonist	56.63	2124
8-OH-DPAT	5-HT_{1A}-specific agonist	73.96	2089
Metergoline	5-HT_{1-2-7} antagonist	90.66	36
Ketanserin	5-HT_2 antagonist	288.18	561
NAN-190	5-HT_{1A}-specific antagonist	>1000	1030
Dopamine		>1000	>10,000

Affinity estimates are given as K_i values in nanomolar concentrations and were determined by a computer-assisted nonlinear curve analysis (GraphPad PRISM 3.0 computer program). [a]Adapted from Angers et al. (1998); [b]adapted from Barbas et al. (2002).

To determine the distribution of 5-HT_{ap1} protein, Angers et al. (1998) performed a Western blot analysis of plasma membrane extracts and RT-PCR amplification of mRNA isolated from various *Aplysia* tissues and found that both the protein and mRNA could be detected in the gill, heart, hermaphroditic duct, kidney, and ovotestis extracts. It is also present in all ganglia of the CNS (Angers et al. 1998). Because 5-HT_{ap1} is present in most *Aplysia* tissue membranes, this receptor might play a role in multiple physiological functions of 5-HT such as reproduction, circadian rhythms, feeding, and modulation of defensive behavior.

The 5-HT_{ap2} Receptor Is Negatively Coupled to Adenylyl Cyclase and Is Exclusively Expressed in the CNS

Membranes isolated from transiently transfected HEK 293 cells with 5-HT_{ap2} receptor bind [^{3}H]LSD in a saturable and dose-dependent manner with an estimated K_d of 4.37 nM (Barbas et al. 2002). The rank order of affinity of various serotonergic agonist and antagonists is given in Table 1. As expected for a 5-HT receptor, 5-HT_{ap2} displayed a higher affinity for 5-HT than for dopamine or octopamine. Its pharmacological profile revealed important differences when compared to those of the mammalian members of the 5-HT_1 subfamily. Methiothepin, a nonselective 5-HT antagonist, showed the strongest affinity (Table 1). The affinity of 5-HT_{ap2} for methiothepin is higher than for any of the cloned mammalian 5-HT receptors. These results suggest that the functions of 5-HT_{ap2} receptor are probably related to those of an ancestral receptor, which existed before the divergence of the 5-HT receptor subtypes in vertebrates, and that it kept characteristics of more than one receptor subtype (5-$HT_{1,5,7}$ receptors). The pharmacological profile of 5-HT_{ap2} is distinct from that of 5-HT_{ap1} (Table 1). It will now be possible to discriminate between these 5HT_1-like receptors in 5-HT-induced physiological responses using appropriate agonists and/or antagonists.

Stimulation of the 5-HT_{ap2} receptor by 5-HT in HEK 293 cells inhibits forskolin-induced cAMP accumulation in a dose-dependent manner (Barbas et al. 2002). The same result was obtained with the agonist 5-CT. In addition, 5-HT produces no inhibition of cAMP accumulation in the presence of 1 nM of the antagonist methiothepin. Pertussis toxin, a known Gi-protein inhibitor, at a concentration of 50 ng/mL, completely blocks the effect of 5-HT, indicating that 5-HT_{ap2} receptor interacts with a pertussis toxin-sensitive G-protein. Interestingly, treatment with pertussis toxin not only inhibits the Gi-dependent decrease of cAMP level, but also seems to reveal a functional coupling between 5-HT_{ap2} and Gs-protein that produces an increase in the cAMP level. Examples of such dual coupling to Gs and Gi pathways have been reported for the α_{2A}- and β_2-adrenergic receptors in mammalian cells (Eason and Liggett 1995; Xiao et al. 1995). Stimulation of the 5-HT_{ap2} receptor can modulate overlapping but distinct sets of signal transduction mechanisms by its interaction with Gi and Gs.

The expression of 5-HT_{ap2} mRNA in various tissues of *Aplysia* was screened by RT-PCR and was found only in the CNS. 5-HT_{ap2} mRNA could be detected in the abdominal ganglion (without the bag cells), in the bag cells, and also weakly in the cerebral and pleural-pedal ganglia (Barbas et al. 2002). The finding that its expression is restricted to the CNS suggests a potential role for the 5-HT_{ap2} receptor in modulating synaptic transmission and neuronal excitability.

5-HT Receptor(s) Coupled to Gs or 5-HT_{apAC}

5-HT receptors that activate adenylyl cyclase, or Gs-coupled receptors, have not yet been cloned in *Aplysia*. One hypothesis would be that, although one or several specific Gs-coupled 5-HT receptors might be expressed in the CNS, the amount of mRNA coding for these receptors is lower than that coding for receptors

that have already been cloned. Therefore the techniques used to preferentially amplify the most abundant cDNA might not be an effective strategy. PCR amplification of SN libraries would help resolve this problem. Another hypothesis would be that the Gi or Gq 5-HT receptors also fulfill coupling to Gs under different conditions of stimulation by 5-HT. This possibility seems unlikely, however, because Cohen et al. (2003) found distinct pharmacological properties for an adenylyl cyclase-coupled 5-HT receptor.

Cohen et al. (2003) recently used biochemical assays to pharmacologically characterize the 5-HT receptor(s) that activates adenylyl cyclase in the *Aplysia* CNS. They also examined the 5-HT-induced cAMP-dependent modulation of the electrophysiological properties of SNs. They identified compounds that are effective in blocking the Gs-coupled 5-HT receptor(s) in *Aplysia* CNS and called this class of receptor 5-HT_{apAC}. Eight of the 14 tested antagonists were effective against 5-HT_{apAC} in CNS membranes. The rank order of potency of these antagonists is given in Table 2, with methiothepin being the antagonist with the highest potency. Comparison of the pharmacological profile of the 5-HT_{apAC} receptor with those of mammalian 5-HT receptor subtypes suggests that it most closely resembles the 5-HT_6 receptor subtype, although two specific 5-HT_6 antagonists (olanzapine and Ro-04-6790) were not effective against 5-HT_{apAC}.

Of the 14 compounds tested, methiothepin was also the most effective in inhibiting 5-HT stimulation of adenylyl cyclase. Methiothepin substantially inhibited two effects of 5-HT on SN firing properties: spike broadening in tetraethylammonium/nifedipine and increased excitability. Consistent with cyproheptadine blocking 5-HT stimulation of adenylyl cyclase, cyproheptadine also blocked the 5-HT-induced increase in SN excitability. In conjunction with other pharmacological probes, Cohen et al. (2003) proposed that the antagonist methiothepin should be useful in analyzing the role of 5-HT in various forms of neuromodulation in *Aplysia*.

Thus far, none of these four cloned *Aplysia* 5-HT receptors have been formally implicated in the molecular processes underlying synaptic facilitation or memory encoding. However, because we know the G-proteins that are potentially coupled to these receptors, the wealth of information on biochemical cascades activated by G-proteins in this and other systems can now help us infer their possible roles in sensitization. These possibilities will now be discussed.

Table 2. Antagonists Tested for Inhibition of 5-HT Stimulation of Adenylate Cyclase in *Aplysia* CNS

Antagonists[a]	Specificity
Methiothepin	Nonselective 5-HT antagonist
Metergoline	5-$HT_{1\text{-}2\text{-}7}$ antagonist
Fluphenazine	D_1/D_2 dopamine receptor antagonist
Clozapine	5-HT_2 antagonist
Cyproheptadine	5-$HT_{1\text{-}2}$ antagonist
Risperidone	5-HT_2 antagonist/D_2 dopamine receptor antagonist
Ritanserin	5-HT_{2A} antagonist/inverse agonist
NAN-190	Selective 5-HT_{1A} antagonist
Inactive antagonists	
GR-113808	Selective 5-HT_4 antagonist
SB-204070	Selective 5-HT_4 antagonist
Olanzapine	5-$HT_{2\text{-}6}$ antagonist
Ro-04-6790	Selective 5-HT_6 antagonist
RS-102221	Selective 5-HT_{2C} antagonist
Spiperone	5-$HT_{2A/1}$ antagonist/D_2 dopamine receptor antagonist

[a]Adapted from Cohen et al. (2003).

Possible Physiological Roles of *Aplysia* 5-HT Receptors

Activation of 5–HT Gs–coupled Receptor(s)

The modulatory effects of activation of a potential 5-HT Gs-coupled receptor in the *Aplysia* nervous system have been studied in considerable detail. Following binding of 5-HT, these receptors activate adenylyl cyclase (AC) and increase the concentration of cAMP in SNs (Fig. 3). In turn, cAMP activates PKA by causing its two regulatory subunits to dissociate from the catalytic subunits, which then phosphorylate and covalently modify a number of target proteins (Ghirardi et al. 1992; Braha et al. 1993; Byrne and Kandel 1996; Angers et al. 2002a). The switch from short- and intermediate-term to long-term synaptic facilitation is initiated by multiple exposures to 5-HT, which induce PKA to translocate to the nucleus where it stimulates gene transcription. There, PKA is thought to lead to removal of the inhibitory action of the repressor cyclic AMP response element binding protein-2 (CREB-2). Some of these nuclear PKA effects could rely on its ability to recruit ERK, which is also translocated to the nucleus, where it is thought to phosphorylate CREB-2 and contribute to the activation of a transcriptional cascade. PKA phosphorylation of a transcriptional activator cyclic AMP response element binding protein-1 (CREB-1) is necessary for its binding to cyclic AMP regulatory elements (CREs), located in the promoter region of cAMP-inducible genes (for review, see Alberini 1999). Because binding of 5-HT to a receptor that engages a G-protein increasing the activity of AC (Gs) is the initial step in this cascade of molecular events, cloning of the 5-HT_{apAC} receptor(s) would give us important information about the molecular mechanisms underlying synaptic facilitation. Expression of these receptors in cell lines would then help identify their functional properties and their possible mechanisms of regulation (binding, time of expression to the membranes, desensitization of the receptor, cross-talk with other pathways, heterodimerization, etc.). It could also allow the synthesis of specific drugs or antibodies capable of blocking their action during sensitization training.

Activation of Two 5-HT Gi-coupled Receptors

Application of 5-HT has generally been associated with an increase in cAMP levels in *Aplysia*. Nevertheless, application of 5-HT also triggers inhibitory responses in *Aplysia* (Jennings et al. 1981; Frost et al. 1988; Fisher and Carew 1993; Ram et al. 1994; Xu et al. 1995; Storozhuk and Castellucci 1999a,b; Bristol et al. 2001). In these cases, binding of 5-HT to Gi-coupled 5-HT receptors in the CNS could be involved. Gi-coupled 5-HT receptors can interfere, by their negative coupling to AC, with pathways that are dependent on cAMP. They might also be involved in modulating PKC or ERK pathways, perhaps by cross-talk between receptors. This phenomenon could be implicated in behavioral plasticity and learning in *Aplysia*.

5-HT receptors that inhibit AC have been implicated in learning and memory processes in mammals: Depending on their cellular localization, they can act by inhibiting the firing rate and/or by decreasing neurotransmitter release (Buhot et al. 1995; Buhot 1997). In mammalian systems, a subset of 5-HT_{1A} receptors, the somatodendritic autoreceptors, reduce cell firing and curtail the synthesis and release of 5-HT (Buhot 1997). Activation of serotonergic autoreceptors in the presynaptic nerve terminal (5-HT_{1D} receptors in human or 5-HT_{1B} receptors in rodents) decreases the local synthesis and release of transmitter (Buhot et al. 1995). Further investigation of the detailed localization of the 5-HT_{ap1} and 5-HT_{ap2} receptors in the *Aplysia* CNS is necessary to determine whether these receptors are also autoreceptors. Identification of the expression patterns at the cellular and subcellular levels should provide additional clues about their role in the modulation of synaptic transmission that occurs dur-

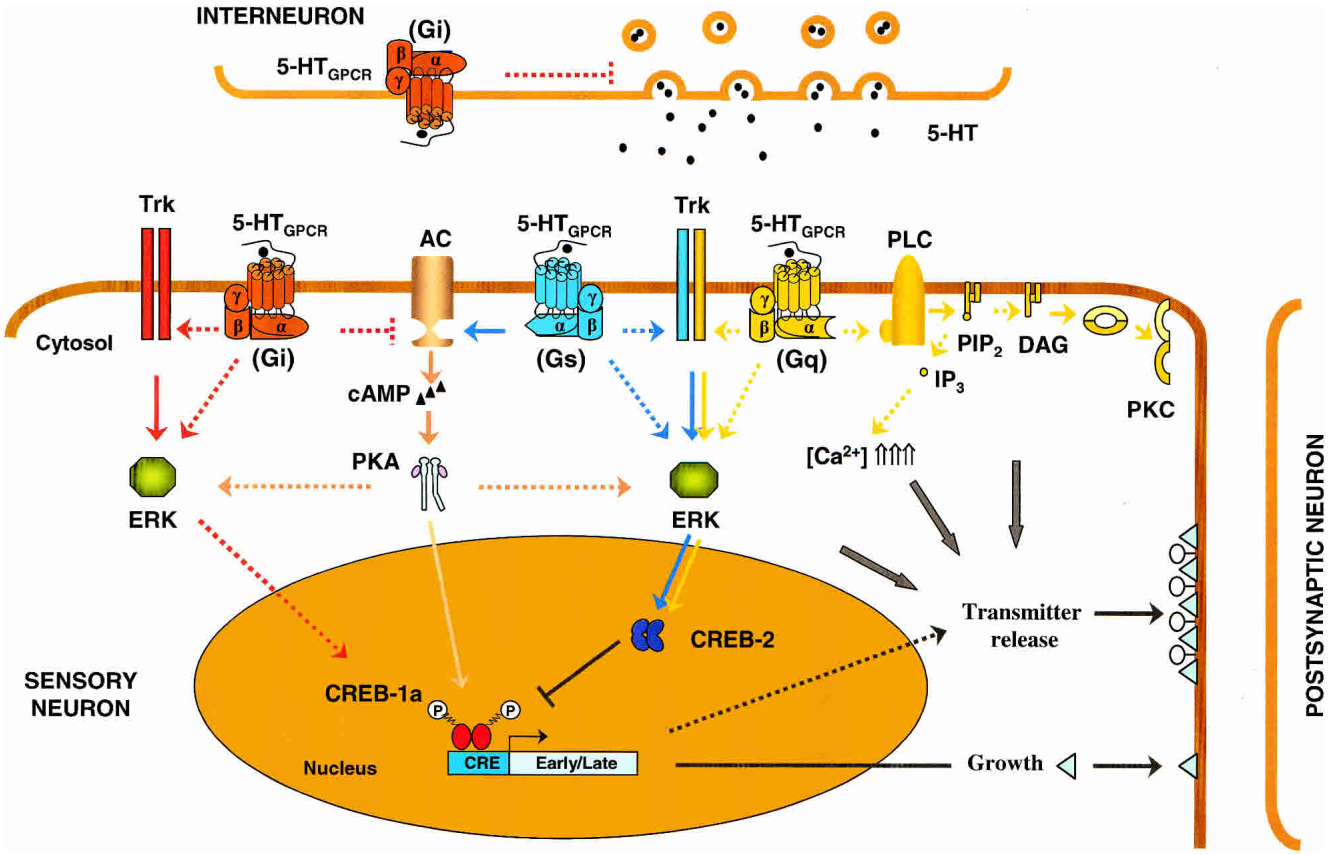

Figure 3 Schematic representation of putative roles of 5-HT receptor modulation of neuronal properties in *Aplysia californica*. The sensory neuron to motor neuron synapse involved in withdrawal reflexes is used as an example. After stimulation, facilitatory serotonergic interneurons release 5-HT that binds 5-HT receptors (5-HT$_{GPCR}$). The final effect of 5-HT may depend on the specific expression patterns of 5-HT receptors and signaling molecules within different cells. Binding of 5-HT to a Gs-coupled receptor stimulates adenylyl cyclase (AC; pink pathway). Activation of the cyclase increases the cAMP concentration and induces activation of the cyclic AMP-dependent protein kinase A (PKA; activation is indicated by an arrow). Activation of PKA can phosphorylate and covalently modify a number of target proteins, including components of the exocytotic machinery of release, to enhance transmitter availability and release. With repeated stimulation, PKA can recruit the extracellular signal-regulated kinase (ERK, MAPK), and can translocate to the nucleus where it phosphorylates the cyclic AMP response element binding proteins (CREBs). Phosphorylation by ERK of the repressor isoform CREB-2 removes its inhibition on CREB-1a. Phosphorylation of CREB-1a induces transcription of early/late genes containing cyclic AMP response elements, leading to growth of new synaptic connections and potentially transmitter release. Gs-coupled receptor might also activate ERK (blue pathway) by transactivation of receptor tyrosine kinases (e.g., Trk, which can be activated by neurotrophins), or by direct activation of the ERK cascade. To turn down the release of 5-HT from interneurons, Gi-coupled receptors might act as presynaptic autoreceptors (inhibition is indicated by ⊣). When expressed at the surface of sensory neurons, Gi-coupled receptors can inhibit the Gs-dependent activation of the cyclase and turn down the cascade (red pathway). Under sustained release of 5-HT, Gi-coupled receptors might complement the activation of the ERK pathway by transactivation of Trks or direct activation of ERK components. Besides the PKA-signal pathway, there is a phospholipase C-PKC signaling pathway activated by 5-HT receptors (beige pathway). Gq-coupled receptor-activated phospholipase C (PLC) produces diacylglycerol (DAG) and inositol triphosphate (IP$_3$) by cleaving the phosphatidyl inositol PIP$_2$. IP$_3$ is water-soluble, and can diffuse into the cytoplasm. There it binds to a receptor on the endoplasmic reticulum to release Ca^{2+} from internal stores. DAG remains in the membrane, where it activates the protein kinase C (PKC). Gq-coupled receptors might also be capable of enhancing the activation of the ERKs. For clarity only, two different sets of Trk receptors and ERK appear on the figure; there is no evidence that Gi-, Gs-, or Gq-activated pathways modulate distinct pools of ERK. Transmitter availability and release can also be dependent on activation of other signaling molecules (e.g., PKC, Ca^{2+}) as shown by gray arrows. Speculative interactions are represented by (- - -). The molecular mechanisms underlying serotonergic modulation of MNs are still poorly understood and were omitted on this diagram.

ing learning and memory. Although, in principle, Gs- or Gq-coupled 5-HT receptors could also decrease transmitter release in *Aplysia*, data from mammalian systems suggest that 5-HT$_{ap1}$ and 5-HT$_{ap2}$ are more likely to be involved in these inhibitory actions, either in serotonergic neurons, where they would act as autoreceptors, or in interneurons, where they could be implicated in the inhibitory action of 5-HT in polysynaptic pathways (Trudeau and Castellucci 1992, 1993).

Activation of Gq-coupled Receptors

Besides the PKA-signaling pathway, a phospholipase C-PKC-signaling pathway can also be activated by 5-HT receptors. Binding of 5-HT to Gq-coupled receptors activates phospholipase C (PLC). PLC then cleaves phosphatidyl inositol PIP$_2$ to produce diacylglycerol (DAG) and inositol triphosphate (IP$_3$). IP$_3$ is water-soluble and diffuses into the cytoplasm, where it binds to a receptor on the endoplasmic reticulum to release Ca^{2+} from internal stores. DAG remains in the membrane where it activates PKC, which is active only when translocated from the cytoplasm to the membrane. Binding of 5-HT to Gq-coupled receptor(s) can activate a Ca^{2+}-dependent PKC in SNs of *Aplysia* (Fig. 3; Sacktor and Schwartz 1990; Sossin and Schwartz 1992). There is no evidence of 5-HT-mediated IP$_3$ production or release of Ca^{2+} from internal stores in sensory neurons after activation of 5-HT receptors. Interestingly, Manseau et al. (2001) demonstrated that the Ca^{2+}-independent form of PKC is very important for facilitation

at depressed synapses. The link between a 5-HT Gq-coupled receptor and the activation of the Ca^{2+}-independent form of PKC is unclear. Because PKC activity contributes to facilitation of neurotransmitter release, modulates K^+ currents, and contributes to spike broadening, the Ap5-HT_{B2} receptor might therefore be involved in modulation of synaptic plasticity (Ghirardi et al. 1992; Sossin et al. 1994; Sugita et al. 1994).

G-protein Regulation of Ion Channels

Many types of neurotransmitter receptors regulate ion channels through activation of G-proteins. The process by which this regulation occurs is best established for Gi-coupled receptors. The 5-HT_{1A} receptors, when coupled to G-protein, activate specific inwardly rectifying K^+ channels in mammalian neurons (Ehrengruber et al. 1997), or inhibit voltage-gated Ca^{2+} channels (Lembo et al. 1997). Regulation of both types of channel most likely occurs primarily through the βγ subunits of G-proteins, which directly open the K^+ channels and limit the opening of Ca^{2+} channels in response to membrane depolarization (Mark and Herlitze 2000; Zamponi 2001). Some evidence also suggests that Gαs-coupled receptors (Drolet et al. 1997) and Gαq-coupled receptors (Simen et al. 2001; Zamponi 2001) modulate the voltage-dependent gating of certain Ca^{2+} channels. In *Aplysia*, 5-HT modulates K^+ and Ca^{2+} channel responses (Klein and Kandel 1980; Siegelbaum et al. 1982; Benson and Levitan 1983; Shuster et al. 1991; Braha et al. 1993; Sugita et al. 1994). This regulation is thought to rely on the activation of PKA and/or PKC pathways. However, it is also possible that direct modulation of ion channels by 5-HT receptors, independently from the activation of downstream messenger cascades, might be involved in the regulation of synaptic transmission that occurs during behavioral modifications in *Aplysia*.

Cross–Talk Between 5–HT Receptors in Aplysia

GPCRs have long been viewed as acting through parallel biochemical pathways. However, a growing amount of evidence now suggests that these pathways are not always independent and that significant interactions can occur between receptors or G-proteins. This phenomenon is usually referred to as "cross-talk."

In *Aplysia*, ERK, a downstream substrate of many tyrosine kinase signaling cascades, has been implicated in the induction of long-term synaptic plasticity (Martin et al. 1997; Zhang et al. 1997). Purcell et al. (2003) recently demonstrated that tyrosine kinase activity is required for both 5-HT-induced long-term synaptic facilitation of SN–MN synapses and tail-shock-induced long-term sensitization. Moreover, both effects of tyrosine kinase activity are mediated, at least in part, through ERK activation. Most importantly, these results suggest that tyrosine kinase activation occurs downstream of the 5-HT receptor, but upstream of ERK activation (Fig. 3). A variety of biochemical mechanisms through which GPCRs activate ERK have been demonstrated (for review, see Lowes et al. 2002; Luttrell 2002). Potential mechanisms for ERK activation are signals initiated by classical G-protein effectors leading to a PKA- and/or PKC-dependent phosphorylation of proteins involved in ERK activation. GPCRs can potentially activate ERK via cAMP (Stork and Schmitt 2002) and DAG (Puente et al. 2000) via a PKA- and/or PKC-independent mechanism. Activation of ERK has typically been attributed to the activation of receptor tyrosine kinase (RTK) in neurons, for example by the epidermal growth factor (EGF) receptors, by the nerve growth factor (NGF) receptors, or by Trk receptors which are activated by neurotrophins (Lowes et al. 2002). Originally, activation of GPCRs and RTKs by extracellular messages was thought to occur independently. Increasing evidence demonstrates that there are complex interactions between each of these pathways. At least two RTKs, platelet-derived growth factor (PDGF) receptors (Linseman et al. 1995; Herrlich et al. 1998) and EGF receptors (Luttrell et al. 1997; Eguchi et al. 1998) can be "transactivated" by GPCRs. Cross-talk between GPCRs and RTKs allows integration of extracellular signals on the ERK cascade. For example, many GPCR agonists have been shown to trigger activation of the ERK cascade through tyrosine phosphorylation of RTKs, leading to a transactivation of these receptors. Direct interaction between β-arrestin and components of the ERK cascade might also be involved in ERK activation (for review, see Lowes et al. 2002; Luttrell 2002). The contribution of various Gα (Gαs, Gαq, and Gαi) and Gβγ subunits for activation of ERK has been reviewed by Gutkind (1998). It is now apparent that GPCRs can activate ERK via intracellular intermediates such as calcium, PKC, cAMP, PKA, phosphatidylinositol 3-kinase (PI3K), or via Gα and Gβγ subunits. It is attractive to think that one way of strengthening the induction and maintenance of memory in *Aplysia* might be through the activation of diverse 5-HT receptors that modulate the ERK pathway, which is involved in the induction of transcription (Fig. 3). One can then imagine a situation where robust stimulation would cause a large release of 5-HT, which would bind to Gi-, Gq-, and Gs-coupled receptors. Activation of these receptors would activate several signaling cascades that would converge on ERK, an essential step for the induction of long-term synaptic plasticity.

The two protein kinase cascades activated by 5-HT, PKA and PKC, are also capable of interacting in *Aplysia* (Byrne and Kandel 1996). For example, Sugita et al. (1997) showed that activation of PKC by phorbol esters specifically attenuated aspects of the 5-HT activation of the cAMP/PKA cascade. Previous studies have demonstrated that PKC interacts with a cAMP/PKA cascade at the level of AC by increasing its activity and consequently the level of cAMP (for review, see Pieroni et al. 1993; Cooper et al. 1995). PKC can also interact with the PKA cascade at the level of the receptor. PKC can phosphorylate various types of receptors bound to ligands, resulting in desensitization and inhibition of the ligand-induced increases in intracellular messengers in mammals (Zhang et al. 1996; Dale et al. 2002). In *Aplysia* neurons, 5-HT binding to Gq- and Gs-coupled receptors could mediate PKC inhibition of 5-HT-induced activation of the PKA pathway, thereby leading to the suppression of 5-HT-induced facilitation at the transition between short- and long-term memory (Sugita et al. 1997). This mechanism would explain why synaptic facilitation induced by long 5-HT exposure requires PKC, whereas short 5-HT pulses necessitate PKA (Braha et al. 1990; Hochner and Kandel 1992; Sugita et al. 1992).

Cross-talk can also occur at the level of the receptors. It was demonstrated that phosphorylation by PKA of some GPCRs switches the coupling of the β_2 adrenoreceptor from Gs to Gi (Daaka et al. 1997; Zamah et al. 2002). Barbas et al. (2002) showed that treatment with pertussis toxin not only inhibits a Gi-dependent decrease of cAMP level by the 5-HT_{ap2} receptor, but also seems to reveal a functional coupling between 5-HT_{ap2} and Gs-protein that produces an increase in the cAMP level. These findings indicate that stimulation of 5-HT_{ap2} can modulate overlapping but distinct sets of signal transduction mechanisms, and can give rise to distinct (or even opposing) physiological functions in *Aplysia* neurons by its dual coupling properties. Thus, 5-HT_{ap2} might be capable of complementing the activation of 5-HT_{apAC} receptors in modulating short-term and long-term memory.

The discovery that GPCRs may form heterodimers that display distinct pharmacological properties raises fascinating possibilities concerning the plasticity and diversity of these signaling systems (for review, see Bouvier 2001; Angers et al. 2002b). 5-HT receptors are capable of forming homodimers when expressed alone and heterodimers when co-expressed (Xie et al. 1999; Lee

et al. 2000). This type of association between different 5-HT receptors suggests the novel possibility that cross-talk may be possible not only at the level of second-messenger cascades, but even at the level of the receptors, adding increasing diversity to the cross-talk at the second-messenger/protein kinase cascade levels. 5-HT receptors might also be capable of interacting with receptors from other neurotransmitters (e.g., dopamine receptors) and thus allow for an unexpected level of functional diversity, by providing a point of interaction between transmission systems.

Overview and Future Directions

In order to understand the specific role of cloned 5-HT receptors in modulation of behaviors and physiological properties in *Aplysia*, localization of the expression of these proteins is necessary. Recently, using in situ hybridization, Barbas et al. (2003) mapped the site of expression of the two Gi-coupled receptors, 5-HT_{ap1} and 5-HT_{ap2}, in the CNS of *Aplysia*. Localization of expression of 5-HT receptors in neurons can also be accomplished by single-cell RT-PCR (Schacher et al. 2000; Vilim et al. 2001) or using antibodies to detect the presence of these proteins at the cell surface. Mapping of the sites of expression for all cloned 5-HT receptors will provide critical information concerning the heterogeneity of 5-HT receptor populations at the surface of presynaptic and postsynaptic neurons, and thus will ultimately allow us to study their roles in memory formation in *Aplysia*. These roles can be further confirmed by using RNAi and/or antisense approaches to abolish expression of specific receptors in identified neurons. For example, the neuronal circuit mediating siphon withdrawal reflex is now rather well understood, and a number of neurons within this circuit are modulated in response to 5-HT or sensitization training. It will now be possible to investigate which 5-HT receptor subtypes are expressed by SNs, interneurons, and MNs and clarify their role in memory processes.

Although exciting progress has been made in the characterization of 5-HT receptors in *Aplysia*, a number of subtypes likely still remain to be characterized. Early pharmacological and electrophysiological studies revealed the presence of at least six 5-HT receptor subtypes. Only four receptors, representing three different subtypes, have been cloned thus far. In particular, the molecular cascades underlying facilitation of SN–MN synapses during sensitization seem to depend on the activation of a cAMP-PKA pathway by a specific Gs-coupled 5-HT receptor (Cohen et al. 2003). This receptor is certainly one important missing link in our knowledge of memory processes in *Aplysia*. The rapid and striking developments of molecular biological techniques that we are witnessing now will provide us with exciting new tools for further characterizing *Aplysia* 5-HT receptors, and thus help us further understand the complexity of the 5-HT neuromodulatory processes set in motion during memory formation.

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