IDENTIFICATION AND FUNCTIONAL ANALYSIS OF CRUSTACEAN

SEROTONIN RECEPTORS

by

NADJA SPITZER

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ABSTRACT

Constantly changing environments force animals to adapt by cycling through multiple physiological states. Plasticity in sensory, motor, and modulatory neural circuits is an essential part of these adaptive processes. Invertebrates with their accessible, identifiable neurons are excellent models for investigating the molecular and cellular mechanisms underlying state-dependent neural plasticity, and provide insight into similar processes in more complex systems. These properties have allowed highly detailed characterization of several crustacean circuits with respect to their connectivities, cellular properties, responses to various inputs, and outputs.

Serotonin (5-HT) is an important neuromodulator in virtually every animal species. 5-HT signals are mediated primarily by a large family of metabotropic receptors on target cells that activate diverse intracellular signaling cascades. Although 5-HT's effects on crustacean circuits have been studied in detail, the mediating receptors have been inaccessible until recently. Crustacean receptors had not been cloned and specific drugs for use in physiological experiments could therefore not be identified. Coupling properties of 5-HT receptor families are strongly conserved between phyla, but pharmacological profiles are not. The extent of pharmacological divergence among invertebrates is unclear, however, as no systematic functional profile of 5-HT receptors

from related species has been determined. This work shows that orthologs of two 5-HT receptors, $5\text{-}HT_{2\beta}$ and $5\text{-}HT_{1\alpha}$, are highly conserved at the molecular, functional and pharmacological level between two distantly related decapod crustaceans, *Panulirus interruptus* and *Procambarus clarkii*.

A suite of drugs was functionally characterized at *Panulirus* and *Procambarus* $5\text{-}HT_{2\beta}$ and $5\text{-}HT_{1\alpha}$ receptors in cell culture, which were then used to investigate the roles of the receptors in pyloric cycle frequency modulation in the stomatogastric ganglion, a model central pattern generator. The two receptor subtypes were found to serve different roles in the circuit and their function depends on the initial state of the circuit.

Finally, an antibody recognizing $5\text{-}HT_{1\alpha}$ was used to map the localization of this receptor within the crayfish nervous system. $5\text{-}HT_{1\alpha}$ is localized to somata and neuropil throughout the nerve cord, suggesting it may respond to synaptic, paracrine or neurohormonal 5-HT signals. The protein and mRNA expression levels are variable between individual animals, perhaps reflecting distinct physiological states.

Index Words: Pharmacology, Physiology, Invertebrate, G protein coupled receptors, Crayfish, Lobster, Plasticity, *Panulirus*, *Procambarus*, Stomatogastric

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2-Me-5-HT	2-methyl-serotonin
5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine
$5\text{-HT}_{1\alpha\text{crust}}$	Crustacean type 1α serotonin receptor
$5\text{-HT}_{1\alpha\text{Pan}}$	Type 1α serotonin receptor from *Panulirus interruptus*
$5\text{-HT}_{1\alpha\text{Pro}}$	Type 1α serotonin receptor from *Procambarus clarkii*
$5\text{-HT}_{2\beta\text{crust}}$	Crustacean type 2β serotonin receptor
$5\text{-HT}_{2\beta\text{Pan}}$	Type 2β serotonin receptor from *Panulirus interruptus*
$5\text{-HT}_{2\beta\text{Pro}}$	Type 2β serotonin receptor from *Procambarus clarkii*
8-OH-DPAT	(±)-8-hydroxy-2-(di-*n*-dipropylamino) tetralin
AB	anterior burster
cAMP	cyclic adenosine monophosphate
DOI	2,5-dimethoxy-4-iodoamphetamine
GPCR	G protein coupled receptor
IC	inferior cardiac neuron
LP	lateral pyloric neuron
lvn	lateral ventricular nerve
MAPK	mitogen-activated protein kinases
mCPP	1-(*m*-chlorophenyl)-piperazine
MeOTryp	5-methoxytryptamine
mvn	medial ventricular nerve
PD	pyloric dilator neuron
pdn	pyloric dilator nerve

PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PY	pyloric neuron
STG	stomatogastric ganglion
stn	stomatogastric nerve
STNS	stomatogastric nervous system
VD	ventricular dilator neuron
α-Me-5-HT	α-methyl-serotonin

Chapter 1 – General Introduction

Physiological systems transition through multiple states on variable time scales

The nervous system must establish and maintain multiple physiological and behavioral states and generate responses that are state-appropriate from the same modulatory stimulus.

All animals must adapt within an environment which is constantly changing. Pressure to change one's physiology or behavior might come from the physical environment, as in seasonal changes, or from activities of the individual such as social interactions. In addition, changes in internal physiological state such as sleep and hunger occur within the animal itself. These changes necessarily occur over a broad temporal range. Responses to sudden stress as experienced from predation are very transient whereas seasonal states incur long-lasting effects on physiological systems. What are the neuromodulatory signals initiating such state transitions? What mechanisms are involved in restructuring the same circuits to produce state-appropriate responses? Which mechanisms within the circuits are changed to generate those responses? Various systems have been examined for state-dependent changes in the structure or configuration of circuits. In addition, many cellular mechanisms of restructuring the responses to neuromodulators have been identified.

Individual animals transition through stable physiological states involving altered signaling processes.

Many relatively long-term state changes occur within the lifetime of a single individual. For example, many animals mate seasonally and experience seasonal changes in hormone levels. In Siberian hamsters, the levels of circulating androgen levels

undergo dramatic seasonal fluctuations resulting in changes in the structure of the medial amygdala and in mating behavior (Cooke, 2006). During hibernation, ground squirrels enter states of torpor that can last up to 3 wks during which they express a specific translational regulator protein, eIF4E. Short bouts of activity (<24hrs) that occur between these sessile periods are characterized by hyperphosphorylation of eIF4E, increasing its activity level and therefore enhancing translational initiation within cells (van Breukelen et al., 2004). State changes can also occur in response to social experience. In mice, for example, aggressive behavior is correlated with increased expression of 5-HT$_{1A}$ receptors in specific forebrain regions (Korte et al., 1996). In anoles, however, increased levels of 5-HT$_{1B/D}$ receptor expression in the brain are observed in subordinate individuals (Baxter, 2001). Individual animals therefore undergo mechanistic transitions that alter their physiology and behavior in a state-dependent manner.

Circadian state changes require regular switching of physiological mechanisms.

Several physiological systems cycle between states on a daily basis with respect to sleep/wake cycles. These systems are controlled through differential activity of specific brain areas. For example, the activity of noradrenergic signaling from the locus ceruleus differs greatly between wakefulness and sleep and is associated with differential state-specific gene expression in the cerebral cortex (Cirelli and Tononi, 2004). Similarly, respiratory rate is depressed during REM sleep through increased cholinergic signaling from neurons in tegmental nuclei (Bellingham and Ireland, 2002).

Short-term state changes occur daily in order to maintain normal function or in adapting to transient perturbations.

Animals live in constantly changing environments and must adapt on a minute-to-minute basis. In rodents, 5-HT_{1A} and 5-HT_7 receptors are both involved in thermoregulation but while 5-HT_7 is thought to mediate fine adjustments in temperature homeostasis, the 5-HT_{1A} receptor is less sensitive and may be involved in hyperthermic responses (Hedlund et al., 2004). Stress responses can be considered as transient state changes that result in short-term alterations of numerous physiological systems within the body. Specific molecular mechanisms are known to underlie these transitions. A short pulse of corticosterone resulting from acute stress immediately but transiently (<24h) activates increased production of interleukin-1β which affects many behaviors (Nguyen et al., 2000). Similarly, a single episode of stress modulates behavior and 5-HT_{2A} receptor expression in hippocampus only transiently (gone 4 days later). Repeated stress, however, and depression-like learned helplessness behaviors are associated with long-term changes in receptor expression in hippocampus as well as in the hypothalamus and frontal cortex (Dwivedi et al., 2005). Short-term state changes can therefore act as an intermediate stage in establishing more stable physiological states.

State-dependent differences in neuromodulatory signaling can act as substrates for evolutionary selection.

Just as repetitive transient changes can give rise to more permanent states, these states can be selected on an evolutionary timescale. In this manner, speciation can be initiated by changes in the environment and in the adaptation of the animals to thrive in that environment. This may result in related species with differentially constructed circuits such that the animals respond differently to the same neuromodulatory stimulus. For example, vasopressin V_{1a} receptors in the brains of the closely related prairie and

montane voles are distributed in species-specific expression patterns. Activation of these
receptors elicits affiliative behavior in monogamous prairie voles and in transgenic mice
with prairie vole-like patterns of expression. The same behavior is not observed with
the same dose of vasopressin in promiscuous montane voles, reflecting adaptation to
a changed socioecological environment (Young et al., 1999). Furthermore, affiliative
behavior is significantly increased in promiscuous meadow voles when V_{1a} receptors are
overexpressed in the ventral pallidum to mimic pairie vole-like patterns of expression
(Lim et al., 2004). Similarly, differential distribution of androgen receptors in the brains
of chickens and quails may be responsible for species-specific differences in crowing
behaviors (Shaw and Kennedy, 2002). Differences in receptor expression patterns
may therefore act as a substrate for evolutionary selective pressures such that the same
modulatory signal elicits species-specific behaviors.

State transitions can be achieved through modulatory input.

What are the signals that initiate and maintain such state-dependent
reconfiguration of systems? The examples above suggest that neuromodulation can act to
restructure circuits, generating specific physiological and behavioral states. As a result,
the same neuromodulatory signals can exert differential effects within those states. What
are the mechanisms underlying these two neuromodulatory roles in circuit plasticity?

**Neuromodulatory mechanisms underlying adaptive transitions between behavioral
states – changes in receptor expression.**
Neuromodulatory signals act as 'gear shifts' in state transitions.

Neurotransmitters are often thought of as synaptically or hormonally released
substances that generate a transient response from target tissues based on activation of
specific receptors in these tissues. In many cases, however, repetitive or maintained

modulatory input can result in more long-term reconfiguration of the target tissues effecting a transition to an alternate behavioral or physiological state. As described above, many state changes are correlated with changes in expression of G protein coupled receptors (GPCRs). A great majority of physiological signals are mediated by GPCRs which share a basic seven transmembrane domain structure (Reviewed in Gether, 2000; Kristiansen, 2004). GPCRs are located in the cell membrane and bind neuromodulators at their extracellular domains or, as in the case for aminergic receptors, in pockets formed by the transmembrane domains. Once bound to a ligand, structural changes in the receptor mediate transmission of a signal that is transferred to multiple effector molecules including heterotrimeric G proteins. Multiple signaling cascades can be initiated by GPCR activation over varying time courses depending on the cell type and its physiological state.

Neuromodulator receptors therefore mediate the signals for restructuring while also being the targets of those configurational changes. Although several modulatory substances have been implicated in restructuring mechanisms, serotonin (5-HT, 5-hydroxytryptamine) has been one of the most extensively studied at both the cellular and systems level. Serotonin is likely an evolutionarily ancient transmitter as it has complex functions in most animals ranging from nematodes to humans (Peroutka and Howell, 1994). In addition, the serotonergic response system is extremely complex, highly plastic and involved in many key physiological processes in diverse species. In many systems, 5-HT itself is the modulatory signal that results in restructuring 5-HT receptors, thus acting as a regulator for its own circuits. What are the mechanisms that underlie such a 'gear shift' effect of neuromodulation?

State-dependent changes in patterns of 5-HT receptor expression.

One way of restructuring the response system for a neuromodulator is to alter the expression patterns of the receptors for that modulator on specific cells or in specific brain regions. Such changes have been observed in correlation with several physiological state changes in various animals. In anoles, subordinate individuals have higher expression levels of 5-HT$_{1B/D}$ receptors in the forebrain than dominants (Baxter, 2001). These receptors are thought to act as both autoreceptors, attenuating release of 5-HT by target cells, and heteroreceptors that act to change release patterns of other modulators. The authors suggest that this leads to inhibition of modulatory signals whose actions in other brain areas elicit aggressive behaviors. Similarly, 5-HT receptor expression is correlated with attack latency in housemice. In this system, however, the circuitry is arranged such that aggressive mice show increased levels of 5-HT$_{1A}$ heteroreceptors in the forebrain compared to mice with longer attack latencies (Korte et al., 1996). Short attack latencies and 5-HT$_{1A}$ expression patterns also correlate with low corticosterone levels and other proactive behaviors indicating that the states of multiple physiological systems may contribute to aggressive behavior. Within a population, mice that have lower resistances to obesity resulting from a chronic high-fat diet have higher levels of 5-HT$_{2C}$ and 5-HT$_{2A}$ receptor expression in the classical satiety center (ventromedial hypothalamus) and olfactory nucleus respectively (Huang et al., 2004). Quantitative RT-PCR analysis of a large number of prefrontal cortex samples indicated that 5-HT$_{2C}$ receptor expression is decreased in schizophrenic patients (Castensson et al., 2003). It is not clear, however, if this change results from the disease itself or from medications taken by the patients. In any case, these examples underline the plasticity of receptor expression patterns and how expression might be regulated to give rise to, or in response to, specific behavioral or physiological states.

Mechanisms underlying changes in expression patterns.

What are the mechanisms that give rise to such differences in expression? In several brain areas 5-HT itself is the signal that causes restructuring of 5-HT receptor populations. A major force in restructuring the 5-HT system is desensitization and/or internalization of specific receptor subtypes in response to treatment with 5-HT or agonist drugs. Down-regulation can occur via multiple mechanisms at different points within the signaling pathway. When this occurs at the level of G protein coupling or signaling, new pathways with different long term cellular effects can be implemented to replace the inhibited signaling cascade. Such restructuring can have profound effects on the physiological state of the cell.

Regulation of the 5-HT$_{1A}$ receptor in response to agonists or antidepressants has been well studied and involves several mechanisms including receptor internalization (Reviewed in Hensler, 2003). Activation of 5-HT$_{1A}$ autoreceptors in the dorsal raphe results in decreased 5-HT release in the prefrontal cortex. These 5-HT$_{1A}$ autoreceptors are internalized within 15 min of agonist (8-OH-DPAT) injection. The reduction of membrane-associated 5-HT$_{1A}$ receptors is maintained for at least 1hr but within 24h the receptors (or new replacements) have returned to the cell surface. This response is not an intrinsic property of these receptors as 5-HT$_{1A}$ heteroreceptors in the hippocampus do not get internalized in response to agonist (Riad et al., 2001). The rates of internalization and recycling mechanisms are therefore specific to certain neuroanatomical structures or cell types. The internalization of 5-HT$_{1A}$ autoreceptors, and GPCRs in general, occurs in a G protein dependent manner (Pucadyil et al., 2004). The Gβγ subunit interacts with the C-terminal of the receptors and is required for phosphorylation of this region of the receptor by G protein receptor kinases (GRKs), the first step in the β-arrestin-mediated internalization cascade (reviewed in Chuang et al., 1996; Lefkowitz and Shenoy, 2005). If internalization of 5-HT$_{1A}$ receptors in response to agonist in the prefrontal cortex is

a homeostatic mechanism, low levels of 5-HT should conversely increase expression of 5-HT receptors in this area. Indeed, postmortem analysis of suicide victims with major depression revealed increased levels of 5-HT$_{1A}$ autoreceptors in the dorsal raphe (Stockmeier et al., 1998). Therefore, the levels of receptor expression in a specific brain area are correlated with the manifestation of a depressed state.

5-HT$_{2A}$ receptors are unusual in that they are down-regulated in response to both agonist and antagonist treatment (Reviewed in Sanders-Bush, 1990). In C6 glioma cells, endogenous 5-HT$_{2A}$ receptors down-regulate in response to 5-HT treatment. This effect involves decreased receptor numbers preceded by reduced receptor mRNA production that is dependent on activation of specific protein kinase C (PKC) isoforms (Anji et al., 2001). In P11 cells, however, 5-HT activation of endogenous 5-HT$_{2A}$ receptors resulted in increased levels of receptor mRNA although receptors on the cell surface are still decreased. It is hypothesized that the mRNA increase here occurs as a compensatory mechanism in response to internalization of surface 5-HT$_{2A}$ receptors. The increase in 5-HT$_{2A}$ mRNA levels is mediated by two distinct pathways, one of which is PKC-dependent while the other is PKC-independent (Wohlpart and Molinoff, 1998b). Similar effects were seen at the level of cell surface receptors when the 5-HT$_{2A}$ was expressed in several different cell lines. Agonist or antagonist treatment caused down- or up-regulation of 5-HT$_{2A}$ receptors depending on the cellular background in which they were expressed (Grotewiel and Sanders-Bush, 1994). Intrinsic properties of specific cells may therefore determine the results of restructuring modulatory signals. Multiple additional varied and complex cellular mechanisms have been implicated in 5-HT$_{2A}$ receptor regulation (Reviewed in Gray and Roth, 2001).

Apart from down-regulation of existing receptor populations and changes in receptor mRNA levels, several additional mechanisms control receptor expression and could be targeted by signals to restructure the response to a transmitter. The

5-HT$_{2C}$ receptor is the only GPCR that is known to undergo mRNA editing. The resulting isoforms display different G protein coupling properties and changed levels of constitutive activity (Reviewed in Sanders-Bush et al., 2003). Interestingly, editing of the 5-HT$_{2C}$ receptor in the forebrain is modulated by serotonergic signaling such that animals depleted of 5-HT express receptor isoforms with higher responses and vice versa (Gurevich et al., 2002).

Most GPCRs have N-linked glycosylations in the N-terminal region that do not affect function but are required for appropriate targeting to the cell membrane. In addition, quality control of glycosylated receptors includes trimming of certain sugar residues as the molecule moves through the endoplasmic reticulum. Appropriate trimming of glycosylations can be critical to normal processing and trafficking of the receptor (Lanctot et al., 2006) and changes in the trimming process could therefore alter the levels of receptor directed to the cell surface. In addition, the β2-adrenergic receptor in higher primates has a unique N-linked glycosylation site in extracellular loop 2. Glycosylation at this site is responsible for directing internalized receptors to lysosomal degradation pathways in response to agonist. As a result, a loss of this glycosylation site eliminates agonist-promoted downregulation of the receptor (Mialet-Perez et al., 2004). State-specific alterations to the enzymatic processes involved in glycosylation and quality control could therefore regulate the number of receptors on the cell surface and thereby alter the cell's sensitivity to a modulator.

Multiple mechanisms therefore contribute to regulating levels of receptor expression. In addition, specific cell types can differ in their use of these pathways. Such plasticity provides numerous differential targets for the establishment of specific and distinct physiological states.

Neuromodulatory mechanisms underlying adaptive transitions between behavioral states – altered receptor signaling properties.

A given GPCR can couple to multiple signaling cascades on several time scales depending on the cell type and the physiological state of the cell. An altered response system, therefore, does not necessarily have to involve changes in expression of receptors at specific sites. Instead, the signaling properties of existing receptor populations could be reconfigured or redefined to effect the 'gear-shift' that underlies a state change.

In cells expressing multiple subtypes of 5-HT receptors, the activation of one population can affect the function of another, a phenomenon termed 'heterologous desensitization'. Such cross-talk between signaling cascades results in differential regulation of 5-HT_{1A} and 5-HT_{1C} receptor activity through phosopholipases activated by 5-HT_2 heteroreceptors. The signaling pathways of 5-HT_{1A} and 5-HT_{1C} receptors are differently modulated by different phospholipases (PLA_2 or PLC) activated by heteroreceptors. Furthermore, although 5-HT_{2A} and 5-HT_{2C} receptors activate PLA_2 and PLC equally, only 5-HT_{2C}-activated (but not 5-HT_{2A}-activated) PLA_2 signaling is able to alter 5-HT_{1B} efficacy. The authors suggest that this specificity might result from compartmentalization of receptor-effector systems within the cell. In addition, differential metabolism of arachidonic acid, a component of the PLA_2 signaling cascade, and the activation state of adenylate cyclase, the modulatory target, can alter the effects of cross-talk regulation (Berg and Clarke, 2001). Similarly, heterologous desensitization of 5-HT_{1A} receptors in specific brain regions resulting from activation of 5-HT_2 receptors occurs at the level of G protein activation, not receptor expression (Valdez et al., 2002). 5-HT_2 receptors activate PKC which could then phosphorylate 5-HT_{1A} receptors, thereby changing their signaling properties (Daaka et al., 1997; Lefkowitz et al., 2002).

Several modifications can alter the signaling strength of a receptor population.

Phosphorylation can change the efficacy of a receptor at specific signaling cascades in addition to initiating internalization and subsequent downregulation (Lembo and Albert, 1995; Chuang et al., 1996). Receptor phosphorylation occurring as a result of the initial signaling cascade can even switch the G protein coupling of a receptor within the same signaling event (Daaka et al., 1997; Lefkowitz et al., 2002). In addition to changing receptor signaling properties, phosphorylation can trigger internalization of receptors (Premont et al., 1995). Short-term alterations in signaling may thereby be converted into more stable reconfiguration of receptor signaling properties.

In addition to alterations in signaling strength of receptors at a second messenger cascade, immense plasticity exists in the structure of those cascades (reviewed in Raymond et al., 2001). This provides for virtually endless configurations that could give rise to multiple net outputs representing different physiological states. More permanent alterations include rearrangement of oligomers with distinct signaling properties (reviewed in Hansen and Sheikh, 2004) and restructuring of scaffolding configurations associated with specific receptors within cellular microdomains (Rich et al., 2001; Zaccolo and Pozzan, 2002). Such changes are often dependent on changes in gene expression, which can result from activation of the receptors themselves via signaling cascades that terminate in activation or suppression of specific transcription factors via MAPK (mitogen-activated protein kinases) pathways (Chen et al., 2002). Changes in the protein scaffold surrounding a receptor can alter the efficacy of coupling and even the identity of the preferred Gα subunit activated. In addition, diverse G protein independent signaling pathways, including those mediated by the G$\beta\gamma$ subunit, are subject to modulation and alteration (Reviewed in Brzostowski and Kimmel, 2001). Altered signaling properties therefore provide numerous targets for state-dependent reconfiguration.

The same neuromodulatory signal can have state-dependent effects.

Circuit restructuring results in different state-dependent effects resulting from application of the same modulator.

One result of receptor system restructuring is that the same modulatory signal can have very different, state-dependent, behavioral effects. Such differences have been observed in several systems that exist in states with varied temporal stabilities. In male Siberian hamsters, for example, the neuroanatomy and expression of androgen receptors in the medial amygdala change seasonally and dictate the ability of androgens to elicit mating behaviors (Park et al., 2004; Cooke, 2006).

Similar state-dependent modulatory effects have been observed in invertebrate systems. In *C. elegans,* 5-HT acts as a switch between two egg-laying states and acetylcholine triggers egg laying events within those states (Waggoner et al., 1998). In the lateral giant tailflip circuit of crayfish, 5-HT has opposing effects on a behavioral output depending on the social experience of the animal. Differential expression of receptor subtype populations may be involved in establishing these state dependent effects, some of which are reversible over a two week period (Yeh et al., 1996; Yeh et al., 1997). Similarly, 5-HT has opposing modulatory effects on pyloric cycle frequency in the stomatogastric ganglion of spiny lobsters (Beltz et al., 1984). Diverse animals therefore draw on the same fundamental mechanisms to effect state changes in modulatory systems.

Crustacean systems as accessible models for investigating mechanisms underlying state-dependent neuromodulation.

Advantage of crustacean systems.

Crustacean systems include several examples of extremely well characterized circuits. These contain large identified neurons which are accessible and whose roles in

eliciting stereotyped behaviors are well understood. Because specific behaviors are often elicited or controlled by single neurons, the cellular mechanisms underlying behavioral state changes can be directly examined in these circuits. Furthermore, since GPCRs and the components of their transduction cascades are evolutionarily well conserved, these mechanisms and the manner in which they are modulated are broadly applicable to more complex systems exhibiting physiological state changes.

Tools to study mechanisms involved in 5-HT modulation are now available for crustaceans.

The physiological effects of 5-HT have been extensively studied in several crustacean circuits, however the receptors mediating these signals have, until recently, been inaccessible. This has changed with the cloning of two crustacean 5-HT receptors, 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ (Clark et al., 2004; Sosa et al., 2004).

The subtypes of 5-HT receptors arose very early in evolution, presumably through gene duplication (Peroutka and Howell, 1994). Representatives of three of the six mammalian groups of 5-HT receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_7$) have been identified in arthropods to date (reviewed in Blenau and Baumann, 2001; Tierney, 2001). Paralogs within each subtype (5-HT$_{1A}$, 5-HT$_{1B}$), however, are believed to have evolved independently after divergence of invertebrate and mammalian evolutionary lines (Peroutka and Howell, 1994; Tierney, 2001). All arthropod amine receptor subtypes characterized to date exhibit the same second messenger coupling as their vertebrate homologs (Witz et al., 1990; Saudou et al., 1992; Colas et al., 1995; von Nickisch-Rosenegk et al., 1996; Blenau and Baumann, 2001; Tierney, 2001; Lee and Pietrantonio, 2003; Chen et al., 2004; Clark et al., 2004). The primary signaling pathway of 5-HT$_1$ receptors results in inhibition of adenylate cyclase via activation of the G$\alpha_{i/o}$ family of proteins. Conversely, 5-HT$_7$ receptors activate adenylate cyclase via Gα_s. Finally, 5-

HT$_2$ receptor subtypes signal through Gα_q proteins to activate phospholipase C resulting in ionositol phosphate and Ca^{2+} signaling within the cell. Three additional G protein coupled 5-HT receptor subtypes, 5-HT$_{4,5\&6}$, have been identified in mammals and may have homologs in invertebrate systems (Tierney, 2001; Clark et al., 2004). As discussed above, however, the signaling generalizations for receptor subtypes are overly simplified (Reviewed in Raymond et al., 2001) and are used primarily in classification of a receptor rather than in predicting the net cellular effects of activating that receptor in its native environment.

As expected, the 5-HT$_{2\beta}$ receptor cloned from *Panulirus interruptus*, the California spiny lobster, activates PLC when expressed in cell culture (Clark et al., 2004). Here we characterized a second receptor, 5-HT$_{1\alpha}$, and compared the signaling properties of both receptors to orthologs from the swamp crayfish, *Procambarus clarkii*. Comparison of two receptor classes from these two distantly related decapod crustaceans should confirm that signaling is conserved for 5-HT receptors within this subphylum.

Functional conservation of crustacean 5-HT receptors

While the basic second messenger coupling is well conserved, the pharmacology of invertebrate 5-HT receptors is variable amongst phyla and in comparison to vertebrate homologs (Tierney, 2001). Using pharmacological agents that have been characterized at mammalian receptors to investigate physiological 5-HT effects in invertebrate systems therefore contributes a degree of uncertainty to interpretation of the results. Comparisons in pharmacological profiles amongst invertebrate receptor orthologs have, however, been made between very distantly related invertebrates (i.e. *Drosophila* vs. *Aplysia*) which may be more evolutionarily distant from one another than from mammals. In addition, these comparisons are based on ligand binding studies that may not reflect the functional efficacy of pharmacological agents. A comparison of receptor orthologs from more

closely related species may prove that receptor pharmacology is generally conserved within evolutionary subphyla. In this work we sought to compare the structural and functional properties of 5-HT receptors from two distantly related decapod crustaceans in order to assess the degree of pharmacological conservation. These comparisons will indicate whether the pharmacological profiles detailed for these crustacean species could be more broadly applicable to related crustacean systems.

Applying crustacean 5-HT receptor tools to investigate state-dependent alterations in model crustacean circuits

The development of specific pharmacological agents and antibodies recognizing crustacean 5-HT receptor subtypes further allowed us to investigate the role that these molecules play in well defined crustacean circuits. The cellular and circuit effects of 5-HT have been detailed in several crustacean nervous systems. For example, 5-HT has modulatory effects on the pyloric cycle frequency in the stomatogastric ganglion of the spiny lobster (Beltz et al., 1984; Flamm and Harris-Warrick, 1986a). Using pharmacological agents whose actions were defined at two crustacean 5-HT receptor subtypes, we were able to investigate the contribution of multiple receptors to the 5-HT effect in individual cells and on the net cycle frequency modulation. In addition, we used an antibody that specifically recognized the 5-HT$_{1\alpha}$ receptor subtype to map the distribution of this receptor in the crayfish central nerve cord. Finally, we investigated plasticity in the expression of this receptor at the protein and mRNA level in the crayfish nerve cord.

The findings from these studies, although performed in relatively simple crustacean systems, are broadly applicable to neuromodulatory mechanisms in more complex systems.

Chapter 2   Characterization of crustacean serotonin receptors

Parts of this chapter have been published:

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* equal authorship; N. Spitzer was responsible for cloning, ICC and confocal imaging of

the crayfish receptor. She also collected confocal images of prawn ICC and edited early

drafts of the manuscript.

Introduction

Serotonin (5-HT) is an important neurotransmitter and neurohormone in most

animal species. Diverse processes are regulated by this biogenic amine including social

behaviors and a variety of systemic physiological functions. Animals have evolved a

suite of receptor types to manage serotonin's multiple effects. To date, seven classes of

serotonin receptors with multiple subtypes have been identified from mammals. These

receptor classes are categorized with respect to their signal transduction mechanisms and

pharmacological properties (Gerhardt and van Heerikhuizen, 1997; Hoyer et al., 2002;

Kroeze et al., 2002)

The relatively simple neural systems of invertebrates have been studied

extensively to investigate the mechanisms underlying aminergic modulation in behaviors

including motor pattern generation (Hooper and DiCaprio, 2004), escape and social

status (Edwards et al., 1999), aggression (Kravitz, 2000) and learning (Kandel and

Schwartz, 1982; Bicker, 1999; Barbas et al., 2003). Serotonin has multiple and complex

modulatory roles in invertebrate nervous systems. In the crayfish, 5-HT has an opposite

net effect on the response of the lateral giant escape circuit to sensory stimulation

depending on the individual's social status (Yeh et al., 1996; Yeh et al., 1997). The rate and concentration of 5-HT application to the circuit also affect this response (Teshiba et al., 2001). In the stomatogastric nervous system of decapod crustaceans, individual identified neurons respond differently to 5-HT application (Flamm and Harris-Warrick, 1986b) and these responses can differ for the same identified neuron in different species (Katz and Tazaki, 1992). These studies suggest that crustaceans express several different 5-HT receptor types and that the relative expression or signaling of these receptors might change in response to social or environmental stimuli. Invertebrate nervous systems are ideal for investigations of the mechanistic basis of modulation and plasticity due to their relatively simple, highly characterized circuits with small numbers of identifiable cells that are responsible for mediating specific behavioral components.

Studies of the mechanisms underlying 5-HT modulation and its regulation in crustaceans have, however, been limited by the lack of accessibility to individual 5-HT receptors in physiological preparations. Recently, steps have been made towards specifically identifying and characterizing crustacean 5-HT receptors with respect to their signaling properties and expression patterns (Clark et al., 2004; Sosa et al., 2004; Spitzer et al., 2005). While the protein sequences and second-messenger couplings of invertebrate 5-HT receptors are relatively well conserved, their pharmacological profiles can vary significantly (Tierney, 2001). In order to study mechanisms mediated by specific 5-HT receptors in crustacean nervous systems, these receptors must therefore be pharmacologically characterized to provide tools for use in physiological preparations. We were interested to know whether 5-HT receptor properties beyond protein sequence are conserved within crustaceans such that drugs with certain actions on receptors from one crustacean species could be used with relative confidence in a physiological preparation from a related species. We therefore characterized and compared two crustacean 5-HT receptors, $5\text{-HT}_{2\beta}$ and $5\text{-HT}_{1\alpha}$, from two distantly related infraorders of

decapod crustaceans, *Panulirus interruptus* and *Procambarus clarkii*, with respect to their protein sequence, signaling mechanisms and pharmacological profiles.

Methods and Materials

Animals

Crayfish, *Procambarus clarkii*, were obtained from Atchafalaya Biological Supply (Raceland, LA) and kept communally at 20°C in 40-liter tanks with continual filtration and aeration.

Chemicals and Cell lines

HEK293, NIH3T3, Cos7 and MDCK cells, EMEM, horse serum, trypsin and penicillin/streptomycin were obtained from American Type Culture Collection (Mannassas, VA). DMEM was from Mediatech Inc. (Herndon, VA). Dialyzed fetal bovine serum (FBS), TRex cell line (293-TR), pDNA4/TO plasmid, blasticidin and zeocin were from Invitrogen (Carlsbad, CA). Cinanserin was obtained from Tocris (Ballwin, MO). All other chemicals were from Sigma (St. Louis, MO). For pharmacology experiments amine and agonist stock solutions (10^{-1}M) were made fresh for every experiment in media or 50% ethanol, respectively. Two exceptions were tyramine which was made fresh as a 10^{-2}M stock in media and methysergide which was made as a 10^{-2}M stock in DMSO and stored at –20°C. Antagonist drugs were made as 10^{-2}M stock solutions in DMSO and stored at –20°C.

Cloning of full-length 5-HT$_{1\alpha}$ and 5-HT$_{2\beta}$ from Procambarus clarkii *and generation of expression constructs*

Complete cloning and sequencing of 5-HT$_{2\beta Pan}$ and 5-HT$_{1\alpha Pan}$ from *Panulirus interruptus* have been previously described (Clark et al., 2004; Sosa et al., 2004).

Total RNA was extracted from the nervous systems of *Procambarus clarkii* as previously described (Baro et al., 1994). First strand cDNA was produced in a standard 20µl reaction using Superscript II (Life Technologies, Gaithersburg), and the reaction buffer and the directions provided with the enzyme: 1X first strand buffer, 1-5µg total RNA, 250ng random hexamer, 500µM dNTP, 10mM DTT.

A large segment of $5\text{-HT}_{1\alpha Pro}$ spanning transmembrane domains III through VII was cloned using nested RT-PCR with degenerate primers (written 5'-3': Fwd1 – GAY GTIIIITGYTGYACIGCIWSNAT; Fwd2 – RTTIRMRTAICCIARCCANARRAA; Rev – CCRAAIARIATICKYTTRAAIGC) based on *Drosophila* 5-HT_1 receptor sequences (Saudou et al., 1992). We completed sequencing of the $5\text{-HT}_{1\alpha Pro}$ cDNA using Rapid Amplification of DNA Ends (SMART RACE cDNA amplification kit, BD Biosciences, Clontech, Cambridge, UK) as previously described (Clark et al., 2004). Constructs containing the complete ORF were assembled using standard procedures (Ausubel et al., 1990). Both strands of the construct were sequenced and errors that had been introduced in the cloning process were corrected with QuikChange Site Directed mutagenesis (Stratagene, La Jolla, CA). The construct was then assembled into the pDNA4/TO (Invitrogen) expression plasmid.

$5\text{-HT}_{2\beta Pro}$ was cloned from crayfish cDNA using degenerate RT-PCR and RACE. Previously, $5\text{-HT}_{2\beta Pan}$ had been identified in the *Drosophila* genome database and the ortholog from *Panulirus* was fully cloned and characterized for signal transduction properties (Clark et al., 2004). Degenerate primers were designed based on conserved regions of these *Panulirus* and *Drosophila* orthologs of $5\text{-HT}_{2\beta Pro}$ (written 5'-3'):

    5-5-1, GAYGTIYTITTYTGYACIGCIWSIATHATG;

    5-5-2, ATGCAYYTITGYACIYTIWSIGTIGAYMGI TT;

    5-3, CATDATDATIARIGGDATRTARAARCA;

    3-5-1, CAYGGIMGIAAYATHMGIATGGARCA;

3-5-2, WUIGARCARAARGCNACNAARGU;

3-3, YUURUURAAIAWIGURUARRA.

Multiple cDNA preparations were used as templates for nested PCR experiments with these degenerate primers to amplify fragments of the crayfish ortholog, as described previously (Baro et al., 1994). Primers specific to 5-HT$_{2\beta Pro}$ were then designed to generate a large clone of 5-HT$_{2\beta Pro}$. The N- and C-terminals of 5-HT$_{2\beta Pro}$ were then cloned using SMART-RACE as described above. A construct containing the complete ORF was assembled, sequenced and inserted into the pIRESneo (Clontech, Mountain View, CA) expression plasmid as previously described for 5-HT$_{2\beta Pan}$ (Clark et al., 2004).

Sequence data was analyzed using Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI). Sequences for other arthropod species were obtained from GenBank and alignments were created to determine sequence identities using the ClustalW algorithm with default settings in Lasergene MegAlign (DNASTAR Inc., Madison, WI). Transmembrane domains were predicted using the TPred program which uses an algorithm based on TMbase, a database of naturally occurring transmembrane proteins (http://www.ch.embnet.org/software/TMPRED_form.html). The snake diagram was generated using the RbDe Web Service available from the institute for computational biomedicine at Cornell University, Ithaca (http://icb.med.cornell.edu/crt/RbDe/Rbde. xml).

Generation of cell cultures expressing crustacean 5-HTRs

HEK293 cells were transfected with a 5-HT$_{2\beta Pan}$ or 5-HT$_{2\beta Pro}$ expression constructs as previously described (Clark et al., 2004). Transiently expressing cultures were used for pharmacological profiling. Briefly, cells were maintained in EMEM supplemented with 10% FBS, 50U/ml penicillin and 50μg/ml streptomycin (normal medium). Cells were plated on 60mm dishes in EMEM without antibiotics, allowed to grow to 95–100%

confluency and then transfected with 2μg of DNA using lipofectamine (Invitrogen). Six hours after transfection, the plates were supplemented to a final concentration of 10% FBS and the medium was replaced with normal medium 24hr after transfection.

In order to functionally characterize 5-HT$_{1\alpha Pan}$ and 5-HT$_{1\alpha Pro}$, we first generated full-length constructs using standard recombinant techniques. The 5-HT$_{1\alpha}$ receptors were first cloned into pIRESneo and stably transfected into several cell lines using lipofectamine as detailed in Results. When these stably transfected lines failed to yield a functional receptor, 5-HT$_{1\alpha Pan}$ and 5-HT$_{1\alpha Pro}$ were cloned into an inducible expression plasmid, pDNA4/TO, behind the tetracycline operator and transfected into 293-TR cells stably expressing the tetracycline repressor protein. Stably transfected cells were selected for >4 weeks in DMEM supplemented with 10% dialyzed fetal bovine serum, 5μg/ml blasticidin and 300μg/ml zeocin (complete medium) (TRex regulated expression system, Invitrogen). Western blots were used to confirm tet-repressor regulated 5-HT$_{1\alpha Pan}$ expression as follows. Cells were plated in 60mm dishes and induced with 1μM tetracycline 6, 8, 12 and 24 hours before collection and isolation of protein by previously described methods (Clark et al., 2004; Sosa et al., 2004). Protein preparations were run on a 10% SDS-PAGE gel, transferred to PVDF membranes and probed with a custom-made rabbit anti-5-HT$_{1\alpha Crust}$ antibody (Sosa et al., 2004). Bands were visualized using Chemiluminescence (Immun-Star, BioRad, Hercules, CA). 5-HT$_{1\alpha Pan}$ production commences within 6h after induction and lasts through 24h of induction (Figure 2-1). Crude protein prepared from lobster nervous system was run as a positive control. Similar results were found for induction of 5-HT$_{1\alpha Pro}$ in stably transfected 293-TR-5-HT$_{1\alpha Pro}$ lines. Based on these findings we induced cells for functional assays for 18-20h (below).
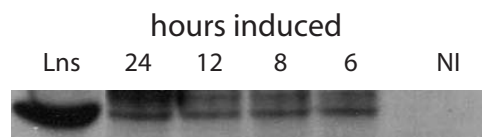
Figure 2-1: 5-HT$_{1\alpha Pan}$ can be stably expressed in an inducible tissue culture system. Western blot with anti-5-HT$_{1\alpha Crust}$ showing expression of 5-HT$_{1\alpha Pan}$ in the lobster nervous system (Lns) and in stably transfected 293-TR-5-HT$_{1\alpha Pan}$ cells after induction with tetracycline for the indicated times (24, 12, 8, 6 hours) and non-induced cells (NI).

Assay of IP release in cells expressing 5-HT$_{2\beta Pan}$ and 5-HT$_{2\beta Pro}$

Inositol phosphate (IP) release was assayed using a slightly modified version of a previously described protocol (Clark et al., 2004). Briefly, transiently transfected cells were split into wells on a 24-well plate with 1μCi/ml of ^{3}H-myoinositol (Amersham, Piscataway, NJ) and allowed to grow to 95-100% confluency over 48 hours. The cells were washed with fresh EMEM and then exposed to 10mM LiCl in EMEM for 20min at 37°C. As applicable, antagonists were added to individual wells and allowed to incubate for an additional 10min. 5-HT or agonist drugs were added to test wells and cells were returned to 37°C for 60min. The medium was removed and replaced with ice cold 20mM formic acid. Plates were then placed on ice for 30min. The cell lysate was collected and applied to AG1-X8 columns (BioRad, Hercules, CA) equilibrated with 20mM formic acid. The columns were washed with 50mM ammonium hydroxide followed by elution of inositol phosphates (IP) with 10ml of 1M ammonium formate/0.1M formic acid. The IP fraction was scintillation counted. Membranes attached to the wells were dissolved in 1M NaOH and scintillation counted as total phosphatidyl inositols (PI). Activation results are expressed as the fraction of radioactivity incorporated in IP over that in IP+PI and normalized to activity observed in negative control wells for every experiment.

[cAMP] determinations in cells expressing 5-HT$_{1\alpha Pan}$ or 5-HT$_{1\alpha Pro}$

Cyclic AMP levels in 293-TR cells stably expressing 5-HT$_{1\alpha Pan}$ or 5-HT$_{1\alpha Pro}$ were determined using a Direct cAMP kit (Assay Designs, Ann Arbor, MI) as previously described (Clark et al., 2004). For receptor activation assays, stably transfected cells were plated in 24-well plates and allowed to grow to 100% confluency. The medium was replaced with 1ml of complete medium containing 1μg/ml tetracycline to induce expression of receptor protein. After 18-20hr the medium was replaced with 1ml of fresh DMEM containing 2.5mM 3-isobutyl-1-methylxanthine to block phosphodiesterase

activity and plates were incubated for 10min. Antagonists were added to individual wells (if applicable) and allowed to incubate for an additional 10min. 5-HT or agonists and forskolin (250nM), a nonspecific activator of adenylate cyclase, were then added to individual wells and left at 37^OC for 30min. The medium was removed and replaced with 0.5ml of 0.1M HCl containing 0.8% Triton X-100. Plates were shaken 30min at room temperature, the lysate collected, centrifuged 5min at 600g and the supernatant assayed for [cAMP] using the Direct cAMP kit and [protein] using a BCA Protein Assay Kit (Pierce, Rockford, Il). Data are presented as picomoles of cAMP per milligram of protein.

Heterologous expression system data analysis

Data for all pharmacology assays involving the heterologous expression systems were plotted and analyzed in GraphPad Prism v.4. Statistics for bar graphs were calculated using a two-way ANOVA with a Bonferroni post-test. Dose-response curves were fitted with a standard slope top-bottom or bottom-top dose-response curve to calculate EC/IC$_{50}$ and efficacy values.

Results

Molecular structure of crustacean 5-HT receptors

The crustacean 5-HT$_{2\beta}$ receptor was recently cloned from *Panulirus interruptus* and characterized with respect to its signaling properties (Clark et al., 2004). Here we used degenerate RT-PCR and RACE to clone the *Procambarus clarkii* ortholog, 5-HT$_{2\beta Pro}$, from crayfish nervous system cDNA. The 5-HT$_{1\alpha}$ receptor was also fully cloned from *Panulirus* and partial sequence was reported for *Macrobrachium rosenbergii* (Sosa et al., 2004). Here we report cloning of the *Procambarus* 5-HT$_{1\alpha}$ ortholog using degenerate RT-PCR and RACE. The predicted amino acid sequences of 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ orthologs

are very well conserved between *Panulirus* and *Procambarus*; 72 and 90% respectively over the entire protein (Figures 2-2, 2-3, Table 2-1).

When comparing 5-HT receptors from various species, the overall sequence identity can fall very quickly, however, the core regions of the protein remain conserved. The extracellular N- and C-terminal domains and the majority of the third intracellular loop are highly variable in 5-HT receptors and are not thought to be critical to signaling or pharmacology (Witz et al., 1990; Saudou et al., 1992; Kroeze et al., 2002). When these variable regions are excluded from the alignment, a core region representing 34 to 59% of the protein remains. This core consists of transmembrane domains and short linker regions important for maintenance of protein structure, ligand binding and signaling. The identity between *Panulirus* and *Procambarus* orthologs in the core protein is very high at 97% for 5-HT$_{2\beta}$ and 98% for 5-HT$_{1\alpha}$ (Table 2-1). The complete 5-HT$_{2\beta}$ sequence from each crustacean is 45% identical to the predicted protein sequence of their ortholog from the fruit fly with an increase to 68% for the core protein. Similarly, crustacean 5-HT$_{1\alpha}$ receptors show 29-53% identity to orthologs from fly, budworm and butterfly with the core protein showing at least 76% identity between any of these five arthropod species. In addition, the cores of both crustacean receptors have greater than 40% identity to their human homologs.

5-HT receptors from all species share key conserved residues responsible for forming the ligand binding pocket in their transmembrane domains. Because the N-terminal and extra-membrane loops of diverse GPCRs are of variable lengths, referring to residues by their absolute position within these proteins does not allow for comparisons between proteins of different species, classes or even splice forms. The Ballesteros-Weinstein numbering scheme used here identifies a crucial and conserved characteristic residue common to all G protein coupled receptors (GPCRs) within each transmembrane domain (TM) that is arbitrarily assigned the number 50 (grey, Figures 2-2, 2-3). Other
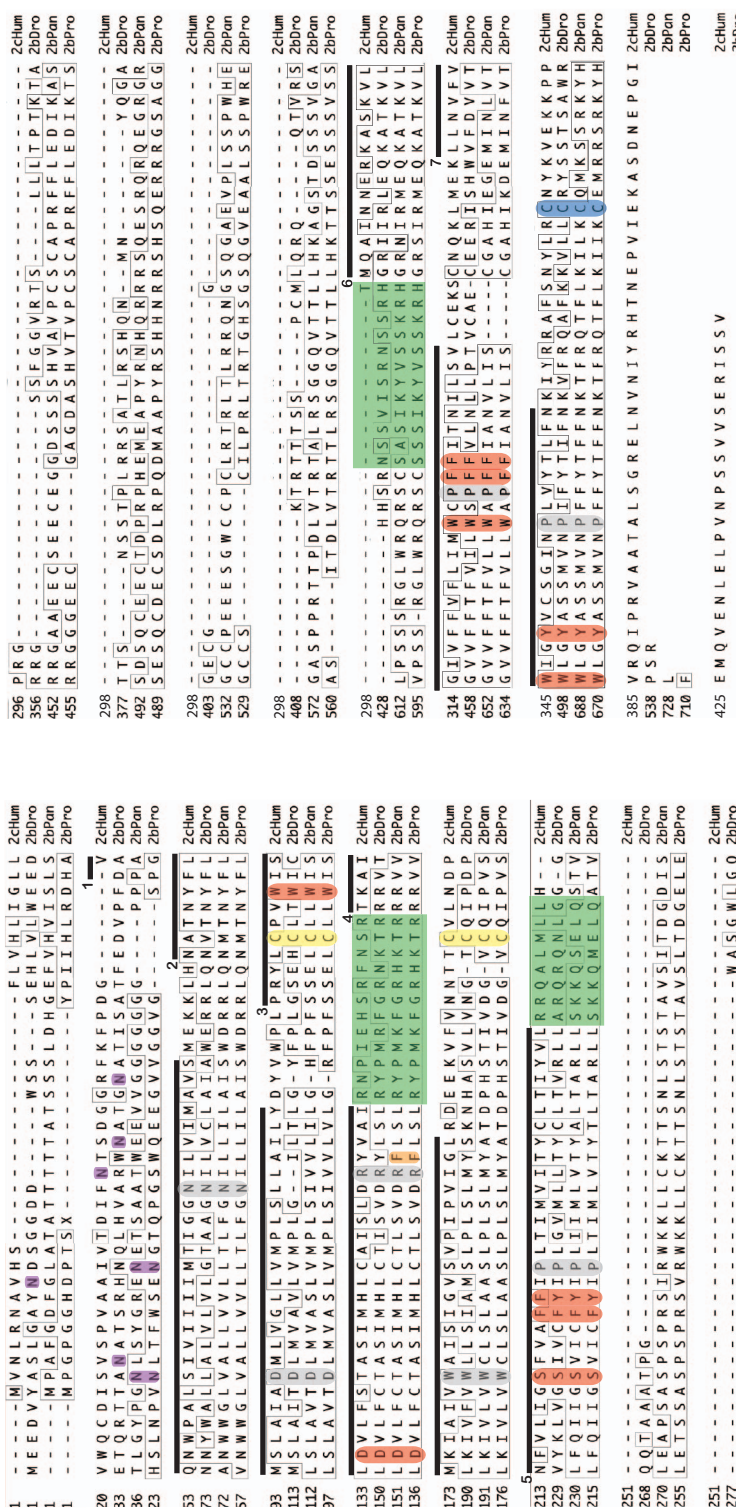
Figure 2-2: 5-HT$_{2\beta}$ receptors contain key structural elements typical of the 5-HT receptor superfamily and are conserved among arthropods. Predicted protein sequences of 5-HT$_{2\beta}$ from *Panulirus* and *Procambarus* are aligned with their ortholog from *Drosophila* and the human 5-HT$_{2C}$ receptor. Residues identical to the 5-HT$_{2\beta Pro}$ sequence have been boxed. Transmembrane domains are indicated with black bars and the reference residue for numbering in each is circled in grey (see Results). Pink: amino acids important for 5-HT ligand binding. Green: areas important for secondary structure. Yellow: consensus sites for N-linked glycosylations. Purple: cysteines involved in forming a bridge important for G protein coupling or activation. Blue: cysteine residue that is often palmitoylated to provide a membrane anchor.
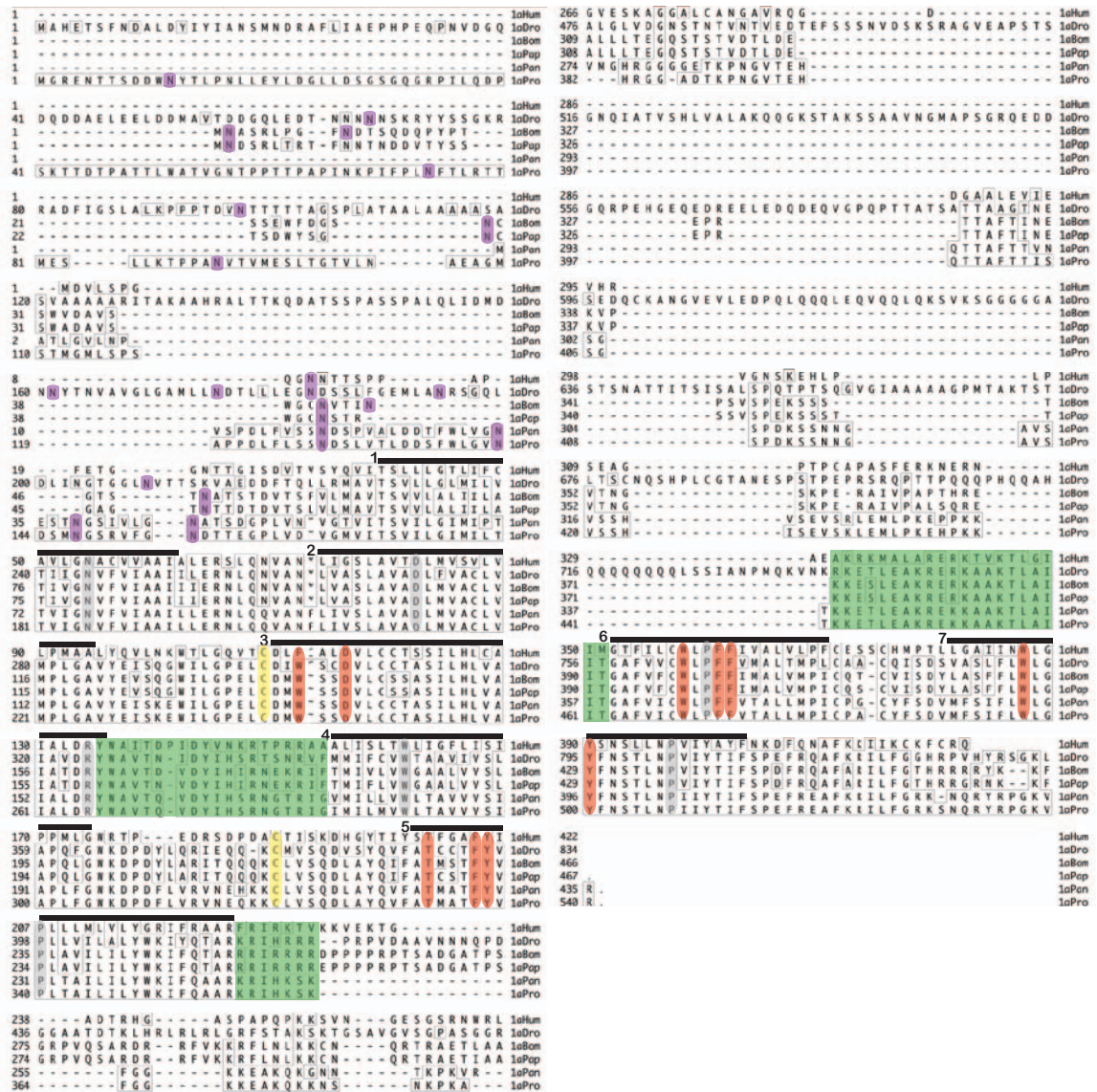
Figure 2-3: 5-HT$_{1\alpha}$ receptors contain key structural elements typical of the 5-HT receptor superfamily and are conserved among arthropods. Predicted protein sequences of 5-HT$_{1\alpha}$ from *Panulirus* and *Procambarus* are aligned with their ortho-logs from *Drosophila, Heliothis* (Bom) and *Papilio* and the human 5-HT$_{1A}$ receptor. Residues identical to the 5-HT$_{1\alpha Pro}$ sequence have been boxed. Transmembrane domains are indicated with black bars and the reference residue for numbering in each is circled in grey (see Results). Pink: amino acids important for 5-HT ligand binding. Yellow: cysteines involved in forming a bridge important for secondary structure. Green: areas important for G protein coupling or activation. Purple: consensus sites for N-linked glycosylations.

Table 2-1

Predicted protein sequences of arthropod 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ receptors are well conserved. Percent identities between 5-HT$_2$ and 5-HT$_1$ receptors from Human, *Drosophila*, *Panulirus* and *Procambarus*. 5-HT$_{1\alpha}$ receptors from two other arthropods, the tobacco budworm *Heliothis virescens* and the swallowtail butterfly *Papilio xuthus*, are also included. Identity was determined for the entire protein and the core region (parentheses) using the CLUSTAL W algorithm in MegAlign (Lasergene). Comparisons amongst 5-HT$_2$ and 5-HT$_1$ receptors are shaded and crustacean ortholog comparisons are in **bold**. Accession numbers are Human (5-HT$_{2CHum}$, NM_000868; 5-HT$_{1A}$, AAH69159), *Drosophila* (5-HT$_{2\beta Dro}$, NP_731257 plus NP_649805; 5-HT$_{1\alpha Dro}$ P28285), *Panulirus* (5-HT$_{2\beta Pan}$, AY550910; 5-HT$_{1\alpha Pan}$, AY528823), *Heliothis* (5-HT$_{1Hel}$, CAA64863), *Papilio* (5-HT$_{1Pap}$, BAD72868).

	2BHum	2βDro	2βPan	2βPro	1αHel	1αDro	1AHum	1αPan	1αPro
2BHum	X	X	X	X	X	X	X	X	X
2βDro	31 (47)	X	X	X	X	X	X	X	X
2βPan	29 (43)	46 (68)	X	X	X	X	X	X	X
2βPro	28 (43)	45 (68)	**72 (97)**	X	X	X	X	X	X
1αHel	21 (33)	21 (35)	16 (34)	16 (34)	X	X	X	X	X
1αDro	22 (33)	25 (37)	17 (33)	18 (33)	60 (81)	X	X	X	X
1AHum	21 (33)	21 (36)	15 (33)	15 (33)	33 (50)	18 (49)	X	X	X
1αPan	22 (34)	21 (36)	14 (31)	15 (32)	53 (77)	29 (76)	36 (50)	X	X
1αPro	22 (34)	22 (36)	14 (31)	14 (32)	53 (77)	30 (77)	37 (49)	**90 (98)**	X
1αPap	21 (33)	21 (34)	16 (33)	16 (34)	92 (98)	35 (82)	37 (50)	56 (76)	53 (77)

residues within that TM domain are then numbered with the TM number followed by the residue number in relation to this identified amino acid (i.e. Phe(6.51) immediately follows the reference residue in TM6, Pro(6.50)) (Ballesteros and Weinstein, 1995). In biogenic amine receptors the charged residue Asp(3.32) is thought to act as a counterion for the protonated amine moiety of amine ligands, agonists and antagonists and is required for ligand binding but not receptor activation (Kristiansen et al., 2000). The presence of Asp(3.32) in combination with Trp(7.40) is considered a unique fingerprint for biogenic and trace amine GPCRs. These amino acids are involved in ligand binding and receptor activation. In addition to these residues, 5-HT receptors typically have a conserved group of hydrophobic amino acids (Trp(3.28), Phe(5.47), Phe(5.48), Trp(6.48), Phe(6.51), Phe(6.52), Trp(7.40), Tyr(7.43)) that form the hydrophobic ligand-binding pocket within the tertiary structure of the receptor (Roth et al., 1997; Kristiansen, 2004 and references therein). The hydroxyl group of 5-HT is thought to be stabilized by the Ser(5.43) residue in transmembrane helix 5. Finally, a disulfide bridge formed between Cys(3.25) and a Cys in extracellular loop 2 (EL 2) is important in maintaining tertiary structure and stabilizing the ligand binding pocket. All of these key amino acids are conserved in 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ from crayfish and lobster (Figures 2-2, 2-3, 2-4).

Crucial residues for Gα subunit binding specificity are located in cytoplasmic amphipathic α-helical extensions of TM5&6 in intracellular loop 3 (IL3) (Figure 2.4, green) (Reviewed in Strader et al., 1995; Gether, 2000; Blenau and Baumann, 2001; Kristiansen, 2004). The C-terminal region of IL3 near the membrane interface with TM6 interacts with the C-terminal end of the Gα protein, conferring the receptor's specificity for a specific Gα subtype. Again, these regions are very well conserved when comparing the crustacean receptor orthologs to each other, to orthologs from other arthropods and to the human homolog (Figures 2-2, 2-3). Although IL2 does not appear to be involved in determining Gα specificity, this loop contains an α-helix that is thought to act in
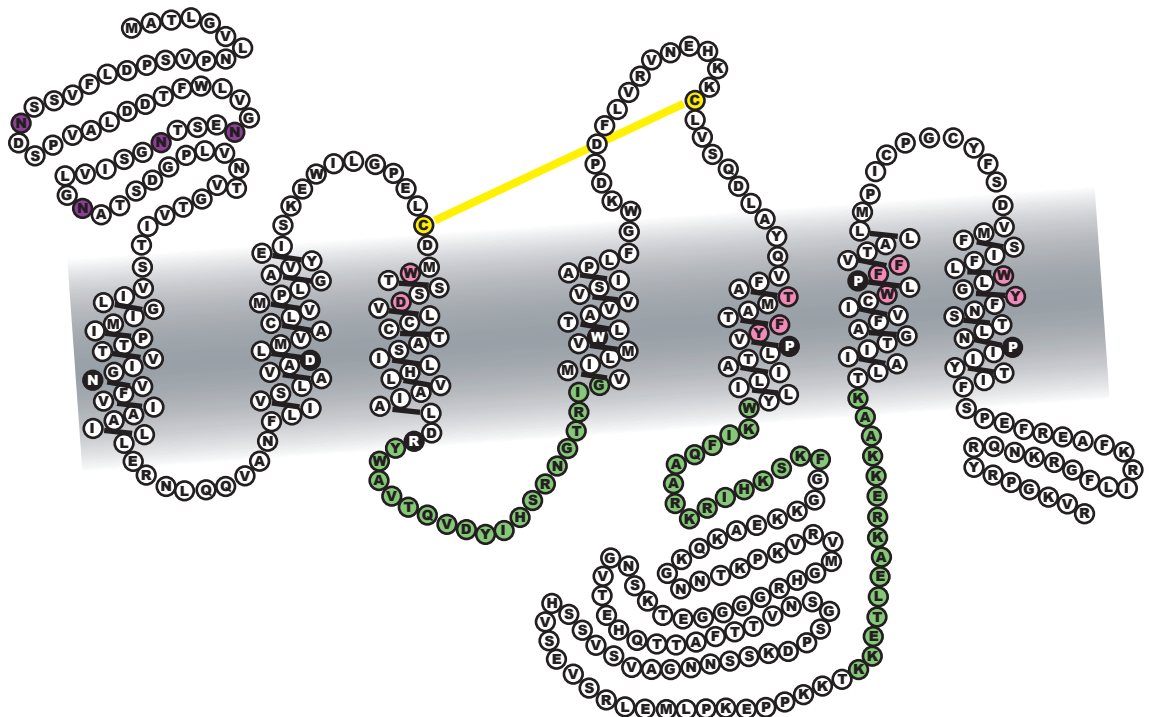
Figure 2-4: Snake diagram of the 5-HT$_{1\alpha Pan}$ receptor showing the putative transmembrane (TM) regions and important residues. The shaded area approximately represents the membrane. Non-membrane regions are coiled to save space and are not meant to represent secondary protein structure. Black residues are highly conserved characteristic residues arbitrarily numbered '50' for each TM domain (see Results). Purple residues are putative N-glycosylation sites. Pink residues are important in forming the ligand-binding pocket. Green areas approximate regions critical to G-protein specificity or activation. Yellow residues and bars indicate a disulfide bridge important for correct receptor conformation.

conjunction with the adjacent DRY motif as a switch between active and inactive states making this loop important for efficient G protein activation. Ligand binding results in protonation of the Asp(3.49) in the DRY motif and in significant rotation of TM3 and TM6 and transition to the active state of the receptor. In addition, the DRY motif and the residues surrounding it are important for constitutive activation in 5-HT receptors (Gether, 2000; Shapiro et al., 2002). Interestingly, the 5-HT$_{2\beta}$ receptor cloned from *Panulirus* has evolved a DRF sequence in place of the DRY that confers increased basal activity of the receptor when stably expressed in cell culture (Clark et al., 2004), this sequence alteration is conserved in 5-HT$_{2\beta Pro}$ from crayfish (Figure 2-2).

Serotonin receptors are extensively post-translationally modified by several mechanisms. Most known GPCRs, including 5-HT$_{2\beta Pro}$ and 5-HT$_{1\alpha Pro}$ have several consensus sites for N-glycosylation (Asn–X–Ser/Thr) in the N-terminal tail (Figures 2-2, 2-3, 2-4) and sometimes in other extracellular regions such as EL2. Proper glycosylation of at least some of these sites is required to obtain appropriate levels of receptor expression on the cell surface (Lanctot et al., 2006). Efficiency of ligand binding and functional activity of receptors are not known to be affected by the glycosylation state in receptors that are expressed in the membrane. Many GPCRs also have a Cys residue in the proximal region of the C-terminal tail that is a putative palmitoylation site; this creates a membrane anchor, generating an additional cytoplasmic loop. This site is present in crustacean 5-HT$_{2\beta}$ receptors but not in 5-HT$_{1\alpha}$ (blue, Figure 2-2). Palmitoylation is important during protein processing to target the receptor to the appropriate site on the plasma membrane and probably facilitates correct association with the G-protein (Qanbar and Bouvier, 2003 and references therein). Palmitoylation of the C-terminal is a reversible post-translational event and could function as a regulatory mechanism; palmitate incorporation and turnover is increased on activated receptors and depalmitoylated receptors are susceptible to hyperphosphorylation. Finally,

receptor phosphorylation, regardless of palmitoylation, is a key event in desensitization or attenuation of a GPCR's signal and is thought to begin with phosphorylation of residues in the C-terminal and third intracellular loop (Lembo and Albert, 1995). The phosphorylated receptor is then bound by β-arrestin, internalized and sorted for either recycling or degradation. Many intracellular and scaffolding proteins are involved in complex interactions that govern regulation of receptor signaling, desensitization and resensitization (reviewed in Chuang et al., 1996; Bockaert et al., 2003; Kroeze et al., 2003; Kristiansen, 2004).

We found that most of the sequence differences between crustacean 5-HT receptor orthologs were localized to the amino terminal and the variable center of the third intracellular loop. To date, no real function has been ascribed to the N-terminal region of 5-HT receptors based on a very limited number of studies (Kroeze et al., 2002). Indeed, in characterizing the *Drosophila* 5-HT$_1$ and 5-HT$_7$ receptors, the amino terminals were cut off to increase expression levels in cell culture with no apparent effect on receptor function (Witz et al., 1990; Saudou et al., 1992). While no function has been immediately obvious in expression systems, the amino terminal can be substantial in size (>100 amino acids in 5-HT$_{1\alpha Dro}$) and may serve an important and highly specified role *in situ*. The variability in this region may contribute to the differences in sensitivity to pharmacological agents observed between 5-HT receptors from the two crustacean species (see below).

The high degree of conservation of the key structures within the crustacean receptors leads us to predict that their signaling pathways will also be the same as those of their arthropod orthologs. Because of the high level of overall conservation, we also might expect the lobster and crayfish orthologs of 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ to exhibit similar pharmacological profiles. In order to compare the functional properties of *Panulirus* and *Procambarus* 5-HT receptors, we heterologously expressed the proteins in cell culture

and used second messenger assays to determine their ligand specificity, signaling and pharmacological properties.

Amine specificity of 5-HT$_{2\beta Pan}$ and 5-HT$_{2\beta Pro}$

The invertebrate 5-HT$_{2\beta}$ receptor was initially cloned from *Panulirus* and shown to be specific for 5-HT over other biogenic amines (Clark et al., 2004). When stably expressed in HEK cells, activation of this receptor resulted in increased intracellular levels of inositol phosphates (IP), activation of protein kinase C (PKC), no change in cAMP levels and constitutive activity conferred by the DRY motif (Clark et al., 2004). In this study we transiently expressed 5-HT$_{2\beta Pan}$ in HEK cells and measured IP release in response to amines and putative pharmacological agents. Interestingly, unlike stably transfected cultures, we found no constitutive activity of 5-HT$_{2\beta Pan}$ when the receptor was transiently expressed (see below).

Non-transfected parental HEK cells did not respond to 1mM concentrations of any of the monoamines in the IP assay (Figure 2-5A). 5-HT$_{2\beta Pan}$ responded to 5-HT, dopamine and tyramine with IP release (Figure 2-5B). The EC$_{50}$ for 5-HT was 52nM while greater than 50µM dopamine (DA) and tyramine (Tyr) were required to activate 5-HT$_{2\beta Pan}$ (Fig. 2-5C). At 1mM these amines also had an efficacy less than 55% that of 5-HT (Table 2-2), indicating that 5-HT is the preferred functional ligand for the 5-HT$_{2\beta Pan}$ receptor. As observed for transiently expressed 5-HT$_{2\beta Pan}$, we found no constitutive activity of transiently expressed 5-HT$_{2\beta Pro}$. 5-HT$_{2\beta Pro}$ responded strongly to 5-HT with a smaller response to DA (Figure 2-5D). The EC$_{50}$ for 5-HT$_{2\beta Pro}$ was 270nM while 1mM DA elicited only a minimal response (Figure 2-5E, Table 2-2).

In the initial characterization of 5-HT$_{2\beta Pan}$, Clark et al. (2004) found that 5-HT was the only biogenic amine to significantly activate this receptor. In this study, however, we have found low levels of activation in response to DA and Tyr. This
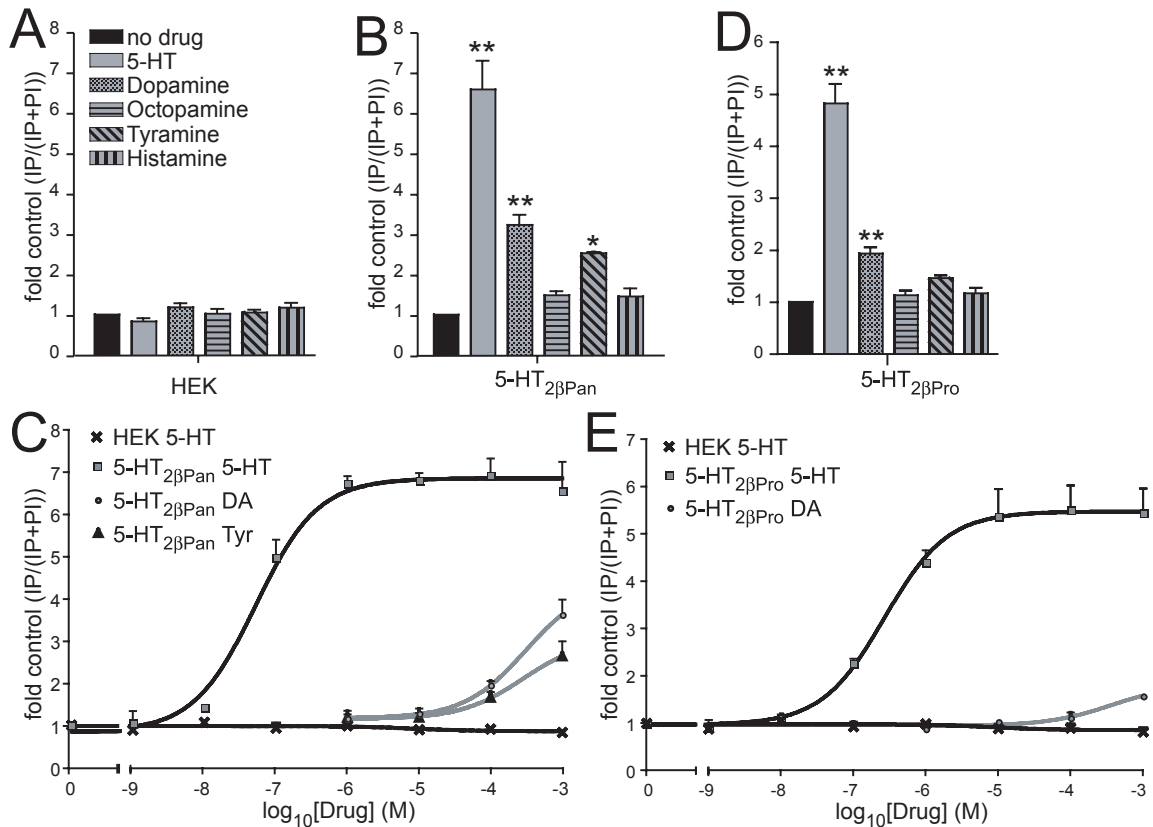
Figure 2-5: 5-HT is the only biogenic amine that acts as a potent agonist at 5-HT$_{2\beta Pan}$ and 5-HT$_{2\beta Pro}$. **A**: Non-transfected parental HEK cells do not show significant IP responses to any of the amines tested. **B**: IP release in response to biogenic amines (10^{-3}M) in cells transiently expressing 5-HT$_{2\beta Pan}$. Cells expressing 5-HT$_{2\beta Pan}$ demonstrate a greater than 6-fold increase in IP release in response to 5-HT and a smaller but significant increase in response to DA and Tyr. Mean±SEM n=3, **p<0.001 and *p<0.05 vs. no drug control. **C**: Dose response curves of 5-HT$_{2\beta Pan}$ to biogenic amines in IP assay. 5-HT$_{2\beta Pan}$ is most sensitive and most effective in response to 5-HT (squares) with an EC$_{50}$ of 52nM. DA (circles) and Tyr (triangles) activate the receptors only at very high concentrations. Non-transfected HEK cells do not respond to 5-HT (crosses). Mean±SEM, n=3. **D**: IP release in response to biogenic amines (10^{-3}M) in cells transiently expressing 5-HT$_{2\beta Pro}$. IP release is increased more than 4 fold in response to 1mM 5-HT in cells expressing 5-HT$_{2\beta Pro}$. A smaller but significant increase in response to Dopamine is observed. Mean±SEM, n=3, **p<0.001 vs. no drug control. **E**: Dose-response curves of 5-HT$_{2\beta Pro}$ to biogenic amines in IP assay. 5-HT$_{2\beta Pro}$ is most sensitive and most effective in response to 5-HT (squares) with an EC$_{50}$ of 270nM. Dopamine (circles) activates the receptor only at very high concentrations. Non-transfected HEK cells do not respond to 5-HT (crosses). Mean±SEM, n=3.

Table 2-2
Agonist profiles of 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ from *Panulirus* and *Procambarus* are very similar. EC$_{50}$ values (Potency) and relative efficacy were calculated from dose-response curves for each drug. Efficacy is presented as a given drug's ability to activate the receptor compared to the maximum activation obtained from 5-HT (100%). Drugs with differential actions on 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ for each species are shown in **bold**. IA: inactive, Bkd: drug has background activity on non-induced cells and was not tested. NS: Curve could not be fit due to complex effects of the drug (see p.44). Abbreviations: DOI, 2,5-dimethoxy-4-iodoamphetamine; 5-CT, 5-carboxamidotryptamine; 2-Me-5-HT, 2-methyl-serotonin; MeOTryp, 5-methoxytryptamine; α-Me-5-HT, α-methyl-serotonin; 8-OH-DPAT, (±)-8-hydroxy-2-(di-*n*-dipropylamino) tetralin; mCPP, 1-(*m*-chlorophenyl)-piperazine.

Drug	Potency (EC50, μM)				Efficacy (%5-HT effect)			
	5-HT$_{2\beta Pan}$	5-HT$_{2\beta Pro}$	5-HT$_{1\alpha Pan}$	5-HT$_{1\alpha Pro}$	5-HT$_{2\beta Pan}$	5-HT$_{2\beta Pro}$	5-HT$_{1\alpha Pan}$	5-HT$_{1\alpha Pro}$
5-HT	0.052	0.27	0.0084	0.031	100	100	100	100
Dopamine	310	362	IA	IA	54	19	IA	IA
Octopamine	IA	IA	IA	IA	IA	IA	IA	IA
Tyramine	283	IA	IA	30	32	IA	IA	67
Histamine	IA	IA	IA	IA	IA	IA	IA	IA
DOI	4.5	IA	Bkd	NS	32	IA	Bkd	NS
5-CT	6.1	4.6	2.2	NS	79	50	100	NS
2-Me-5-HT	0.78	5.2	Bkd	0.043	96	104	Bkd	73
MeOTryp	1.0	1.5	4.2	NS	80	29	94	NS
N-acetyl-5-HT	IA	IA	IA	NS	IA	IA	IA	NS
Quipazine	**IA**	**IA**	**Bkd**	**111**	**IA**	**IA**	**Bkd**	**92**
α-Me-HT	1.5	7.3	1.1	0.22	79	76	120	67
8-OH-DPAT	0.27	1.1	7.6	65	77	64	105	68
mCPP	**IA**	**IA**	**139**	**109**	**IA**	**IA**	**97**	**70**
Methysergide	0.11	0.11	0.089	0.42	48	19	81	109

could have significant implications in a physiological context as DA and Tyr could nonspecifically activate 5-HT receptors. Because relatively high levels of DA and Tyr are required to activate 5-HT$_{2\beta Pan}$, this is likely to be a concern only at synaptic sites where concentrations can reach up to 1mM and not at sites exposed to neurohormonal levels of amines. The difference between the two studies may be due to our measuring IP release (i.e. phospholipase Cβ (PLCβ) activity) whereas the previous study measured PKC activity. Because PKC is downstream of IP release and dependent on the consequent release of Ca^{2+}, the low levels of PLCβ activation by DA and Tyr may not be sufficient to initiate a cascade culminating in PKC activation. Alternatively, the difference may stem from our using transiently transfected cells while Clark et al. (2004) studied stable transfectants. In addition, 5-HT$_{2\beta Pan}$ was found to be constitutively active when stably expressed in HEK293 cells even when measuring PLCβ activity (Clark et al., 2004). However, in our transient transfections with 5-HT$_{2\beta Pan}$ and 5-HT$_{2\beta Pro}$ we found no such constitutive activity. The pathways underlying this activity may, therefore, require establishment of signaling cascades or activation of transcriptional programs that occur over a longer period of receptor expression (weeks) than is permitted in a transient system (days).

In summary, 5-HT$_{2\beta Pan}$ and 5-HT$_{2\beta Pro}$ are specifically activated by 5-HT and couple to the IP signaling pathway with EC$_{50}$ values of 52 and 270nM respectively. Using an antibody-based G protein activation assay M. Clark determined that this signaling occurs via the traditional Gα_q pathway (Spitzer et al., 2004).

Amine specificity of 5-HT$_{1\alpha Pan}$ and 5-HT$_{1\alpha Pro}$

This is the first report of functional characterization for a crustacean 5-HT$_1$ receptor, 5-HT$_{1\alpha}$ from *Panulirus* and *Procambarus* (5-HT$_{1\alpha Pan}$ and 5-HT$_{1\alpha Pro}$). We were not able to express 5-HT$_{1\alpha Pan}$ or 5-HT$_{1\alpha Pro}$ in traditional systems such as HEK293,

NIH3T3, MDCK or Cos-7 cells; all cells that produced the receptor protein, as determined by western blot analysis, were unhealthy and did not grow beyond three weeks. In the few cases where a stable cell line was generated, $5\text{-HT}_{1\alpha}$ protein could not be detected by western blot analysis, suggesting rearrangements in the DNA construct. We do not understand why mammalian cell lines were unable to stably express $5\text{-HT}_{1\alpha}$ using traditional methods. $5\text{-HT}_{1\alpha Pan}$ and $5\text{-HT}_{1\alpha Pro}$ are constitutively active (below) so high levels of expression of the receptors in standard expression systems may have resulted in toxicity. Alternatively, the cell's protein synthesis, export or turnover machineries may have been overly taxed by high levels of $5\text{-HT}_{1\alpha}$ expression such that they were not able to maintain normal functions.

In order to functionally characterize $5\text{-HT}_{1\alpha Pan}$ and $5\text{-HT}_{1\alpha Pro}$ we therefore employed an inducible expression system. $5\text{-HT}_{1\alpha Pan}$ or $5\text{-HT}_{1\alpha Pro}$ constructs were stably transfected into 293-TR cells expressing the tetracycline repressor protein. In this system the $5\text{-HT}_{1\alpha}$ construct is under control of the Tet operator sequence which binds the repressor protein in the absence of tetracycline thereby preventing expression of $5\text{-HT}_{1\alpha}$. Upon addition of tetracycline to the media, the repressor protein dissociates and $5\text{-HT}_{1\alpha}$ is transcribed and translated into protein. The western blot in Figure 2-1 indicates that non-induced cells (tetracycline absent) did not express detectable levels of $5\text{-HT}_{1\alpha Pan}$. After induction (tetracycline present) we were able to obtain high levels of $5\text{-HT}_{1\alpha Pan}$ expression within 6 hours that lasted for at least 24 hours (Figure 2-1). Similar results were obtained for cells induced to express $5\text{-HT}_{1\alpha Pro}$ (not shown).

Induced cells expressing either $5\text{-HT}_{1\alpha Pan}$ or $5\text{-HT}_{1\alpha Pro}$ showed an increased sensitivity to forskolin, a non-specific activator of adenylate cyclase, compared to non-induced cells (Figure 2-6A, C). This supersensitization of adenylate cyclase is typical of cells expressing a constitutively active Gi/o-coupled receptor (Johnston and Watts, 2003). Constitutive activity has been observed in mammalian 5-HT_1 receptors as well as other
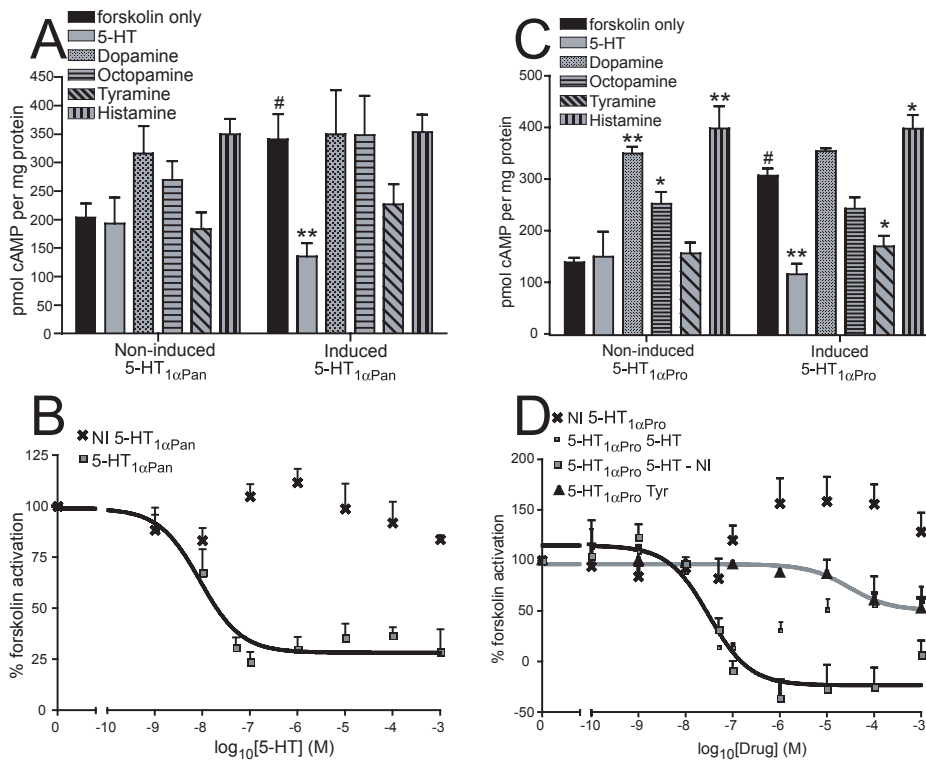
Figure 2-6: 5-HT is the only biogenic amine that acts as a potent agonist at 5-HT$_{1\alpha Pan}$ and 5-HT$_{1\alpha Pro}$. **A**: Inhibition of forskolin-stimulated cAMP accumulation in response to biogenic amines (10^{-3}M) in cells induced to express 5-HT$_{1\alpha Pan}$. Non-induced cells do not significantly respond to biogenic amines (left). Induced cells accumulate significantly higher levels of cAMP in response to forskolin only (black bars). In induced cells adenylate cyclase is significantly inhibited in response to 1mM 5-HT but no other biogenic amines (right). Mean±SEM, n=3, #p<0.05 vs. non-induced, **p<0.001 vs forskolin only. **B**: Dose-response curve of 5-HT$_{1\alpha Pan}$ (squares) to 5-HT. The EC$_{50}$ of 5-HT at 5-HT$_{1\alpha Pan}$ is 8.4nM. Non-induced cells do not show significant changes in forskolin stimulated cAMP accumulation in response to 5-HT (crosses). Mean±SEM, n≥ 3. **C**: Inhibition of forskolin stimulated cAMP accumulation in response to amines (10^{-3}M) in cells induced to express 5-HT$_{1\alpha Pro}$. Adenylate cyclase in non-induced cells is activated by Dopamine and Histamine (left). Induced cells accumulate significantly higher levels of cAMP in response to forskolin only (black bars). In induced cells adenylate cyclase is significantly inhibited in response to 1mM 5-HT but no other biogenic amines (right). Mean±SEM, n=3, #p<0.001 vs. non-induced, **p<0.001 and *p<0.05 vs forskolin only. **D**: Non-induced cells show a positive response of cAMP levels at high concentrations (crosses). Due to this background activity, the dose-response curve of 5-HT$_{1\alpha Pro}$ (small squares) to 5-HT is biphasic. When the activity of non-induced cells in response to 5-HT is subtracted from the response of induced cells, a sigmoidal dose-response curve is obtained (large squares). This curve shows an EC$_{50}$ of 5-HT at 5-HT$_{1\alpha Pro}$ of 31nM. Even at high concentrations tyramine has a minimal effect on 5-HT$_{1\alpha Pro}$ (triangles). Mean±SEM, n≥3.

G protein coupled receptors (Liu et al., 1999; Cosi and Koek, 2000; Johnston and Watts, 2003; Berg et al., 2005).

All known vertebrate and invertebrate 5-HT$_1$ receptors inhibit adenylyl cyclase resulting in decreased cAMP levels after stimulation with forskolin, a nonspecific activator of adenylate cyclase (Tierney, 2001; Hoyer et al., 2002). As expected for a 5-HT$_1$ receptor expressed in a HEK293 cell line, 5-HT$_{1\alpha Pan}$ couples negatively to adenylate cyclase, presumably via Gi/o, resulting in inhibition of forskolin-stimulated cAMP accumulation in response to 5-HT (Figure 2-6A). At 1mM concentrations, 5-HT was the only monoamine to significantly activate 5-HT$_{1\alpha Pan}$ (Figure 2-6A). 5-HT is a highly effective ligand at 5-HT$_{1\alpha Pan}$ with an EC$_{50}$ of 8.4nM (Figure 2-6B, Table 2-2). No significant change in cAMP levels was observed with any biogenic amine in non-induced 293-TR-5-HT$_{1\alpha Pan}$ cells (Figure 2-6A).

Similarly, 5-HT$_{1\alpha Pro}$ activation with 1mM 5-HT also blocks forskolin-stimulated cAMP accumulation. Tyr was the only other biogenic amine that resulted in a significant decrease of cAMP in cells expressing 5-HT$_{1\alpha Pro}$ (Figure 2-6C). Serotonin and tyramine are therefore the only amines that significantly activate the 5-HT$_{1\alpha Pro}$ receptor to inhibit adenylate cyclase. Histamine caused a significant increase of cAMP levels in induced and non-induced 5-HT$_{1\alpha Pro}$ cells. Adenylate cyclase was also activated by DA and octopamine in non-induced cells. Non-induced 293-TR-5-HT$_{1\alpha Pro}$ cells also gave a positive cAMP response at high 5-HT concentrations (Figure 2-6D). During the selection period this cell line appears to have initiated expression of an endogenous 5-HT receptor which is positively coupled to adenylate cyclase. It is not unusual for cell cultures to change their karyotypes or expression profiles over time due to the lack of selection pressure to maintain a constant genome. Such changes can be significant and can lead to laboratory-specific net signaling effects for the same protein (Clark and Baro, in review)(Friedman et al., 2002). Cells that were induced to produce 5-HT$_{1\alpha Pro}$ and treated

with increasing doses of 5-HT responded with an initial decrease in forskolin-induced cAMP mediated by 5-HT$_{1\alpha Pro}$ that was then dampened and partially reversed by activation of the endogenous receptor (Figure 2-6D). Subtraction of the non-induced cell response from the 5-HT curve obtained from induced cells results in a sigmoidal dose-response for 5-HT at 5-HT$_{1\alpha Pro}$ with an EC$_{50}$ of 31nM (Figure 2-6D). Tyramine was an inefficient agonist and slightly reduced cAMP accumulation only at high concentrations (Figure 2-6D) indicating that 5-HT is the preferred functional ligand for 5-HT$_{1\alpha Pro}$.

In summary, *Panulirus* and *Procambarus* 5-HT$_{1\alpha}$ receptors preferentially respond to 5-HT over other biogenic amines with EC$_{50}$ values of 8.4 and 31nM respectively. Other arthropod orthologs of 5-HT$_{1\alpha Pan}$ and 5-HT$_{1\alpha Pro}$ have been cloned and characterized from *Drosophila* (5-HT$_{1ADro\&1BDro}$, originally 5-HT$_{dro2A\&B}$) and *Boophilus microplus* and these show comparable EC$_{50}$ values of 30, 18 and 83nM for 5-HT, respectively (Saudou et al., 1992; Chen et al., 2004). Activation of arthropod 5-HT$_{1\alpha}$ receptors, including those described here, results in inhibition of forskolin-stimulated cAMP accumulation presumably via a Gi/o mediated pathway. In addition, the crustacean 5-HT$_{1\alpha}$ receptor shows constitutive activity when heterologously expressed in HEK cells.

Agonist drugs of crustacean 5-HT receptors

In order to determine if the conservation in sequence and signaling extends to the receptors' responses to pharmacological agents, we tested crayfish and lobster 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ with a suite of agonist drugs. All drugs that showed significant activity at 10^{-3}M in an initial overview were tested in dose-response curves (not shown) which were then used to determine the potency and efficacy (% of maximum 5-HT response at highest concentration) of the drugs. The EC$_{50}$ is a measure of the potency of a drug and reflects its binding affinity at the receptor. Because the maximum effect, or efficacy, achieved by any drug is dependent on the number of receptors expressed we ran a parallel

dose-response curve for 5-HT in every experiment and normalized all agonist maximum effects to the maximum 5-HT response, set at 100%. These data are summarized in Table 2-2. In some cases the agonist had a significant effect on the parental HEK (5-HT$_{2\beta}$) or non-induced (5-HT$_{1\alpha}$) cell lines, presumably acting on endogenous receptors, in which case the drug was not examined further with that line. For example, DOI did not significantly affect the 5-HT$_{2\beta Pan}$ parental HEK cell line, but did have a significant effect on non-induced 5-HT$_{1\alpha Pan}$ cells. Thus, the effect of DOI was examined for 5-HT$_{2\beta Pan}$ but not 5-HT$_{1\alpha Pan}$.

5-HT$_{2\beta}$ agonists

Most of the drugs that activated 5-HT$_{2\beta Pan}$ had an efficacy close to that of 5-HT (Figure 2-7A, Table 2-2). 5-HT$_{2\beta Pan}$ was most sensitive in response to 5-HT with an EC$_{50}$ of 52nM while most of the functional agonists display EC$_{50}$ values ranging from 100nM to 10μM. The following agonist potency rank profile was determined for 5-HT$_{2\beta Pan}$: 5-HT > methysergide > 8-OH-DPAT > 2-Me-5-HT > MeO-tryptamine > α-Me-5-HT > DOI > 5-CT. While most agonists achieved above 75% of 5-HT activation levels, methysergide and DOI were only partial agonists of 5-HT$_{2\beta Pan}$ with efficacies of less than 50% of the 5-HT effect. No change in IP level was detected after application of 10^{-3}M N-acetyl-5-HT, quipazine, or mCPP. None of the drugs had significant effects on non-transfected parental HEK cells (Figure 2-7A).

The agonist profile of 5-HT$_{2\beta Pro}$ was very similar to that of its lobster ortholog (Figure 2-7A, Table 2-2). The rank potency of effective agonists at 5-HT$_{2\beta Pro}$ was methysergide > 5-HT > 8-OH-DPAT > MeOTryp > 5-CT > 2-Me-5-HT > α-Me-5-HT. When both potency and efficacy are considered, however, 5-HT was the strongest ligand at 5-HT$_{2\beta Pro}$. The potency of 5-HT at 5-HT$_{2\beta Pro}$ was five times lower than at 5-HT$_{2\beta Pan}$. Similarly, the potency of α-Me-5-HT and 8-OH-DPAT were lower at 5-HT$_{2\beta Pro}$ than at
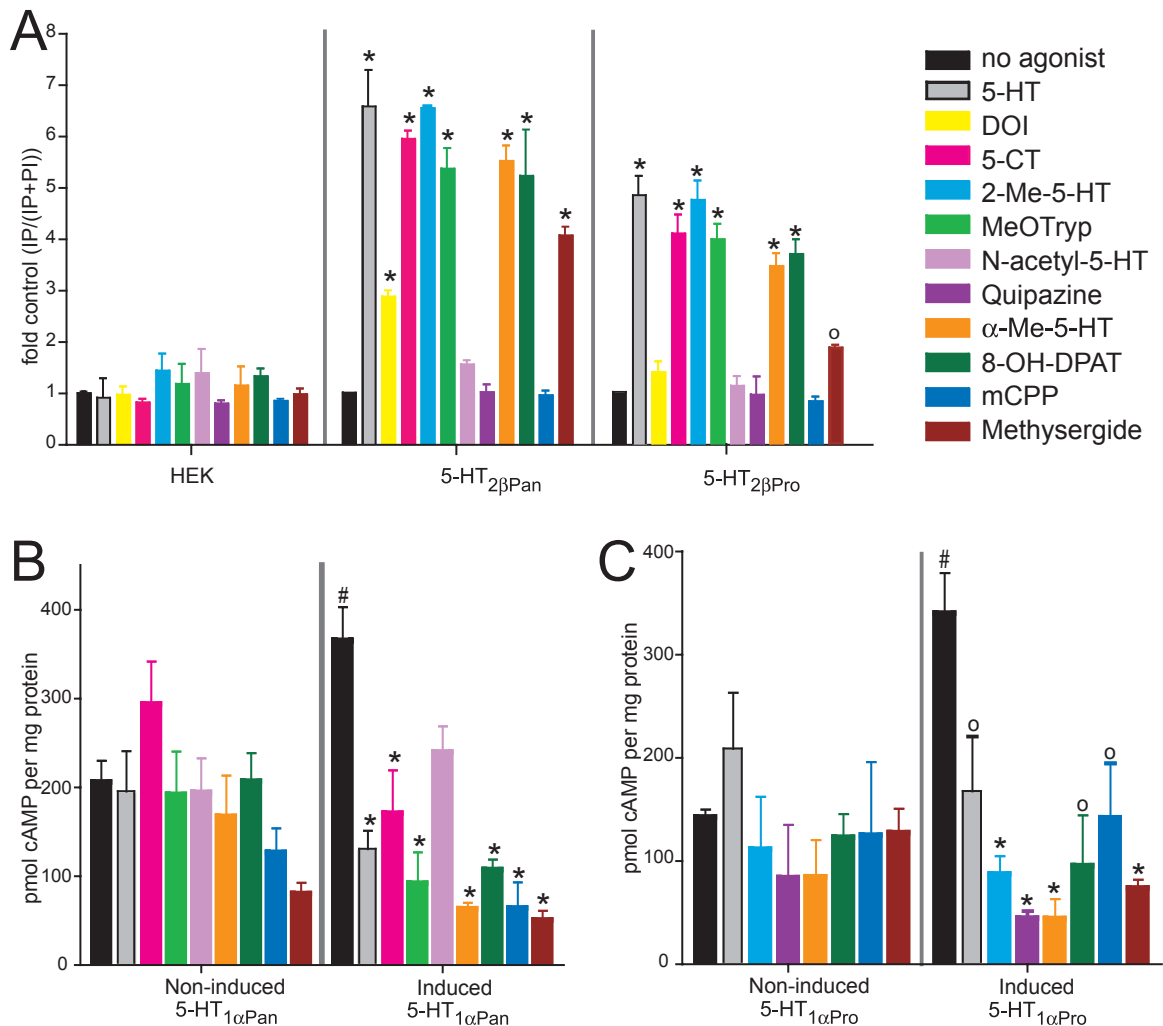
Figure 2-7: Specific drugs have differential agonist activity at 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$. **A**: Release of IP in cells expressing 5-HT$_{2\beta Pan}$ (center) or 5-HT$_{2\beta Pro}$ (right) in response to various putative agonist drugs (10^{-3}M except methysergide, 10^{-5}M). Non-transfected HEK cells (left) show no IP release in response to the drugs. Mean±SEM, n≥3, *p<0.001 and op<0.05 vs no drug. **B**: Inhibition of forskolin stimulated cAMP production in response to the same putative agonists in cells induced to express 5-HT$_{1\alpha Pan}$. Mean±SEM, n≥3, #p<0.01 vs non-induced, *p<0.001 vs forskolin only. **C**: Inhibition of forskolin-stimulated cAMP production in response to the same drugs in cells induced to express 5-HT$_{1\alpha Pro}$. Mean±SEM, n=3, #p<0.05 vs non-induced, *p<0.001 and op<0.05 vs forskolin only.

$5\text{-HT}_{2\beta Pan}$. The crayfish ortholog did not respond to DOI which was a relatively weak agonist of $5\text{-HT}_{2\beta Pan}$. In contrast, the potency of methysergide was the highest of all the drugs tested and equal at the two orthologs. The dose response curve of this drug, however, reached a maximum plateau at only 19% of 5-HT's efficacy at $5\text{-HT}_{2\beta Pro}$, less than half of its efficacy at $5\text{-HT}_{2\beta Pan}$ (Table 2-2). Similarly, 5-CT and MeOTryp, both highly efficient agonists of $5\text{-HT}_{2\beta Pan}$, had similar potencies at $5\text{-HT}_{2\beta}$ from lobster and crayfish but elicited 50% or less of the response obtained from 5-HT at the crayfish ortholog. Conversely, while the efficacy of 2-Me-5-HT was equal to that of 5-HT on both crustacean $5\text{-HT}_{2\beta}$ receptors, it was less potent at $5\text{-HT}_{2\beta Pro}$. Therefore, while the pharmacological profiles of $5\text{-HT}_{2\beta Pro}$ and $5\text{-HT}_{2\beta Pan}$ are conserved in terms of which drugs are active, $5\text{-HT}_{2\beta Pro}$ was consistently less sensitive to agonist stimulation.

$5\text{-HT}_{2\beta Pan}$ and $5\text{-HT}_{2\beta Pro}$ are the only orthologs of this receptor that have been cloned from arthropods. A paralog of this receptor from *Drosophila*, $5\text{-HT}_{2\alpha Dro}$ (originally 5-HT_{2Dro}, see Tierney, 2001; Clark, 2004) binds strongly to α-Me-5-HT, 2-Me-5-HT, ritanserin and methysergide (Colas et al., 1995), all of which we found to be functionally active at $5\text{-HT}_{2\beta Pan}$ and $5\text{-HT}_{2\beta Pro}$. N-acetyl-5-HT and ketanserin, however, were completely inactive at crustacean $5\text{-HT}_{2\beta}$ but have very high binding constants at $5\text{-HT}_{2\alpha Dro}$. When making such comparisons it is important to note that effective binding of a drug to a receptor does not necessarily reflect that drug's functional properties at the receptor. The two receptor paralogs may therefore be more or less similar, functionally, than suggested by these comparisons.

$5\text{-HT}_{1\alpha}$ agonists

Most of the putative agonists tested (1mM) resulted in some activation of the $5\text{-HT}_{1\alpha Pan}$ receptor (Figure 2-7B, Table 2-2). As stated above, some agonists (DOI, 2-Me-5-HT, quipazine) that were tested at $5\text{-HT}_{2\beta}$ had nonspecific effects on non-induced 293-

TR-5-HT$_{1\alpha Pan}$ cells and could therefore not be tested in dose-response curves on induced cells. α-Me-5-HT had a higher efficacy than the natural ligand at 5-HT$_{1\alpha Pan}$ while the other drugs had greater than 80% of 5-HT's efficacy. However, 5-HT was still the most potent of the agonists with an EC$_{50}$ (8.4nM) at least one order of magnitude lower than any other agonist at 5-HT$_{1\alpha Pan}$. Methysergide was significantly more potent than the other agonist drugs with an EC$_{50}$ of 89nM. The relative potencies of functional agonists at 5-HT$_{1\alpha Pan}$ were: 5-HT > methysergide > α-Me-5-HT > 5-CT > MeOTryp > 8-OH-DPAT > mCPP. 5-HT$_{1\alpha Pan}$ did not respond significantly to 10^{-3}M N-acetyl-5-HT.

In general, the agonist profile of 5-HT$_{1\alpha Pro}$ was very similar to that of its lobster ortholog (Figure 2-7C, Table 2-2). The rank potency of effective agonists at 5-HT$_{1\alpha Pro}$ was 5-HT > 2-Me-5-HT > α-Me-5-HT > methysergide > tyramine > 8-OH-DPAT > mCPP > quipazine. The agonist 2-Me-5-HT was almost as potent as 5-HT at 5-HT$_{1\alpha Pro}$, however it showed only 73% of 5-HT's efficacy. 5-HT$_{1\alpha Pro}$ was more sensitive than 5-HT$_{1\alpha Pan}$ in potency to α-Me-5-HT but less sensitive to 8-OH-DPAT although both had a lower efficacy at the crayfish 5-HT$_{1\alpha}$ ortholog. The potency of mCPP was comparable at 5-HT$_{1\alpha}$ from *Panulirus* and *Procambarus* though it was less efficient at the crayfish ortholog. Conversely, methysergide was less potent but had a higher efficacy at 5-HT$_{1\alpha Pro}$ compared to 5-HT$_{1\alpha Pan}$. Two drugs that could not be tested at 5-HT$_{1\alpha Pan}$, 2-Me-5-HT and quipazine, are effective agonists of 5-HT$_{1\alpha Pro}$. Several drugs (DOI, MeOTryp, N-acetyl-5-HT, 5-CT) had complex effects on induced 293-TR-5-HT$_{1\alpha Pro}$ cells that could not be fitted with standard dose-response curves (not shown). These complex effects are likely due to endogenous 5-HT receptors expressed by the non-induced cell line as observed in the 5-HT dose-response curve above. We were therefore not able to determine an EC$_{50}$ or relative efficacy measurements for these drugs.

These data show that, in general, agonist activity at crustacean 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ orthologs is quite conserved. Two agonists that would differentiate between 5-HT$_{2\beta}$ and

5-HT$_{1\alpha}$ were identified. mCPP activates 5-HT$_{1\alpha}$ but not 5-HT$_{2\beta}$ in both *Procambarus* and *Panulirus* but the evolutionary conservation of this specificity is currently unknown because this drug has not been tested in other arthropod systems. Quipazine is also specific to 5-HT$_{1\alpha Pro}$ over 5-HT$_{2\beta Pro}$ and, similarly, is inactive at 5-HT$_{2\beta Pan}$. Unfortunately, quipazine had nonspecific effects on 293-TR-5-HT$_{1\alpha Pan}$ cells and could therefore not be tested on the fourth receptor. Quipazine binds weakly to a molluskan 5-HT$_1$ receptor (Sugamori et al., 1993) and to 5-HT$_{2\alpha}$ from *Drosophila* (Colas et al., 1995) indicating that its specificity may not be well conserved beyond crustacean receptors. The 5-HT$_{1\alpha}$ ortholog from *Drosophila* has been pharmacologically characterized using a radioligand binding assay (Saudou et al., 1992). The only agonist reported to bind 5-HT$_{1\alpha Dro}$ was 8-OH-DPAT, an effective agonist of both 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ from crustaceans, which bound 5-HT$_{1\alpha Dro}$ with a K$_i$ approximately 2.5 times that of 5-HT. 8-OH-DPAT was long thought to be a specific mammalian 5-HT$_1$ agonist but was later found to also activate 5-HT$_7$ receptors (Bard et al., 1993; Sprouse et al., 2004) and it is functionally active at *Drosophila* 5-HT$_{7Dro}$ (Witz et al., 1990). Interestingly, methysergide was an effective agonist of all four crustacean receptors described here. This drug is a functional antagonist of the *Drosophila* 5-HT$_{7Dro}$ receptor (Witz et al., 1990) and of vertebrate 5-HT$_2$ receptors but has agonist activity at some vertebrate 5-HT$_1$ receptors (Silberstein, 1998).

Antagonist drugs of crustacean 5-HT receptors

Because pharmacological agents can be active at multiple 5-HT receptors, strategic combinations of drugs will be necessary to identify the receptors involved in physiological 5-HT effects. In addition to agonists, we therefore tested a suite of putative antagonists on 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ from *Panulirus* and *Procambarus*.

Antagonists were applied to cells 10min before 5-HT application and second messenger assays were used to test receptor activation. Antagonists were first screened

at 10^{-5}M and then dose response curves were generated for any drugs that significantly blocked 5-HT activation of second messengers at that concentration. The IC_{50} was calculated and is reported as a measure of potency for the drug. The efficacy, or maximum effect, for each drug again depends on receptor expression levels and is therefore reported as a percent reduction from the level of receptor activation achieved by 5-HT alone in the same experiment.

5-HT$_{2\beta}$ antagonists

Putative antagonists had no effect on parental HEK cells (Figure 2-8A). Several antagonists (10µM) significantly reduced the amount of IP release in response to 5-HT (1mM) in HEK cells expressing 5-HT$_{2\beta Pan}$ (Figure 2-8A, Table 2-3). The rank potency of effective antagonists at 5-HT$_{2\beta Pan}$ was (+)butaclamol > ritanserin > methiothepin > cinanserin > clozapine. Ritanserin and (+)butaclamol, however, were only partially effective in inhibiting activation of 5-HT$_{2\beta Pan}$ by 5-HT even at the highest antagonist concentration. Of the antagonists tested, ketanserin, spiperone, prazosin, (-)butaclamol, gramine and atropine had no effect at 10^{-5}M.

An overview of antagonist drugs revealed that the same five drugs that blocked 5-HT activation of 5-HT$_{2\beta Pan}$ are also effective at 5-HT$_{2\beta Pro}$ (Figure 2-8A) with a rank potency order of (+)butaclamol > methiothepin > ritanserin > cinanserin > clozapine (Table 2-3). The overall relative potency profile of active antagonists was nearly conserved between 5-HT$_{2\beta}$ from the two species but four of the five drugs had a lower IC_{50} at the crayfish ortholog. In addition, all of the drugs blocked the crayfish receptor to more than 80% while their efficacy at the lobster receptor ranged from 48 to 84%. 5-HT$_{2\beta}$ from lobster and crayfish diverge by almost 30% in the variable regions and these differences could contribute to the increase in potency and efficacy of antagonists at 5-HT$_{2\beta Pro}$.
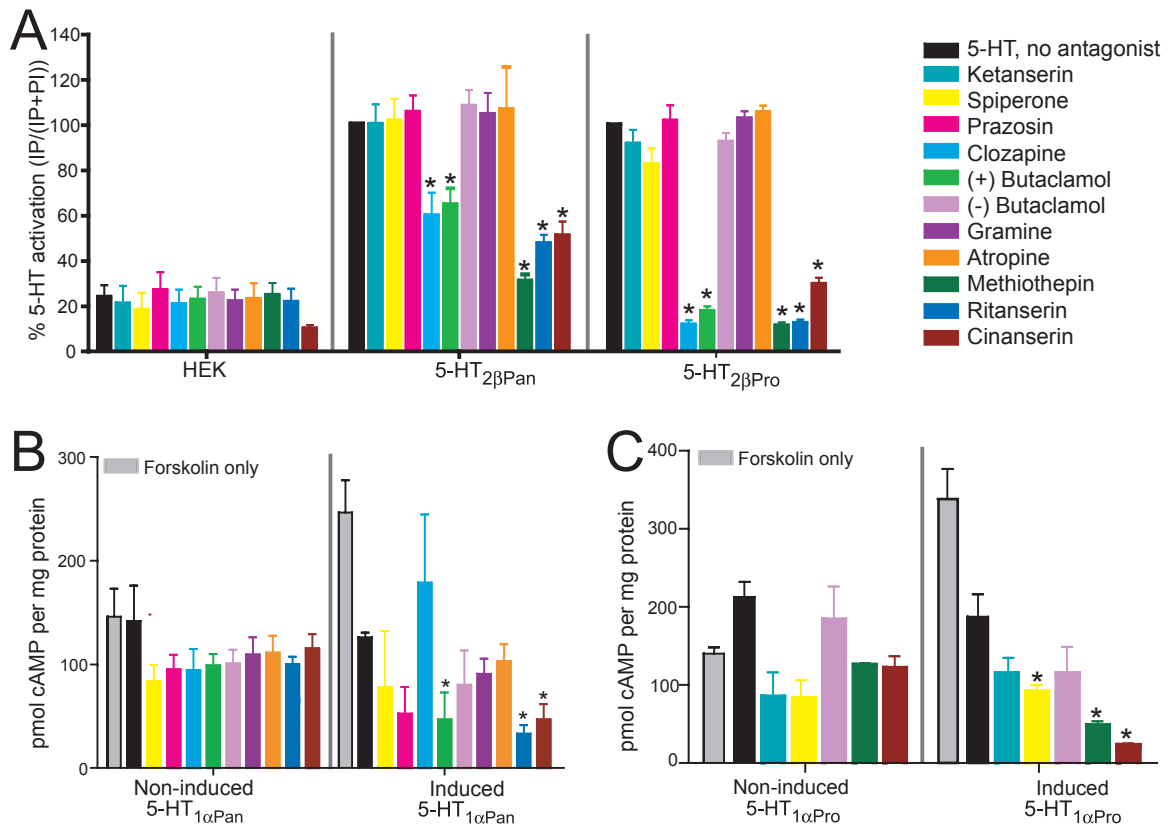
Figure 2-8: Identification of antagonists of 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$. **A**: Putative antagonists have no effect on the parental HEK cell line (left). Several of the antagonists (10^{-5}M) block 5-HT (1mM) stimulated increases in IP levels in cells expressing 5-HT$_{2\beta Pan}$ (center). The same drugs block 5-HT activation of 5-HT$_{2\beta Pro}$ (right). Mean ±SEM, n≥3, *p<0.05 vs 5-HT only. **B**: Antagonist drugs have no significant effect on uninduced 5-HT$_{1\alpha Pan}$ cells (left). None of these drugs (10μM) blocks inhibition of adenylate cyclase by 10^{-5}M 5-HT in cells induced to express 5-HT$_{1\alpha Pan}$ (right). Some putative antagonists actually increase the efficacy of the 5-HT effect. Mean ±SEM, n≥3, *p<0.05 vs 5-HT only. **C**: Some putative antagonist drugs have no significant effect on uninduced 5-HT$_{1\alpha Pro}$ cells (left). None of these drugs (10^{-5}M) blocks inhibition of adenylate cyclase by 10^{-5}M 5-HT in cells expressing 5-HT$_{1\alpha Pro}$ (right). Some putative antagonists increase the efficacy of the 5-HT effect. Mean ±SEM, n=3, *p<0.05 vs 5-HT only.

Table 2-3

Antagonist profiles of 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ from *Panulirus* and *Procambarus* are well conserved. IC$_{50}$ values (Potency) and relative efficacy were calculated from dose-response curves for each drug. Efficacy is presented as the % reduction of the total effect obtained from 5-HT in the absence of antagonist. Drugs with differential actions on 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ for each species are shown in **bold**. IA: inactive, Bkd: drug has background activity on non-induced cells and was not tested, ND: not determined.

	Potency (IC50, µM)				Efficacy (%reduction)	
Drug	5-HT$_{2\beta Pan}$	5-HT$_{2\beta Pro}$	5-HT$_{1\alpha Pan}$	5-HT$_{1\alpha Pro}$	5-HT$_{2\beta Pan}$	5-HT$_{2\beta Pro}$
Clozapine	6.8	2.5	Bkd	Bkd	79	86
Ritanserin	**0.57**	0.18	**IA**	Bkd	58	86
Methiothepin	0.66	**0.097**	Bkd	**IA**	**84**	**88**
(+)Butaclamol	**0.14**	0.017	**IA**	Bkd	48	82
Cinanserin	**1.2**	**1.6**	**IA**	**IA**	**73**	**88**
Gramine	IA	IA	IA	Bkd	IA	IA
(-)Butaclamol	IA	IA	IA	IA	IA	IA
Ketanserin	IA	IA	IA	IA	IA	IA
Spiperone	IA	IA	IA	IA	IA	ND
Prazosin	IA	IA	IA	Bkd	IA	IA
Atropine	IA	IA	IA	Bkd	IA	IA
Chlorpromazine	ND	ND	IA	IA	ND	ND
Flupenthixol	ND	ND	IA	Bkd	ND	ND
Domperidone	ND	ND	IA	Bkd	ND	ND
Fluphenazine	ND	ND	IA	Bkd	ND	ND
Haloperidol	ND	ND	IA	IA	ND	ND
Metoclopride	ND	ND	IA	IA	ND	ND
(-)Sulpiride	ND	ND	IA	Bkd	ND	ND
WAY100635	ND	ND	IA	IA	ND	ND
Yohimbine	ND	ND	IA	IA	ND	ND
S(-)Propanolol	ND	ND	Bkd	IA	ND	ND
SB269970	ND	ND	IA	IA	ND	ND
Metergoline	ND	ND	Bkd	Bkd	ND	ND
Cyproheptadine	ND	ND	Bkd	Bkd	ND	ND
SB224289	ND	ND	IA	Bkd	ND	ND
BRL15572	ND	ND	IA	IA	ND	ND
TFMPP	ND	ND	IA	IA	ND	ND
SCH23390	ND	ND	Bkd	Bkd	ND	ND

5-HT$_{1\alpha}$ antagonists

All of the putative antagonists that were characterized at 5-HT$_{2\beta}$ were also tested on 5-HT$_{1\alpha Pan}$ (Figure 2-8B, Table 2-3). In addition to these, 11 other putative antagonists (10^{-5}M) were tested for their ability to inhibit 5-HT$_{1\alpha Pan}$ activation by 5-HT. None of these drugs was able to significantly block the inhibition of cAMP accumulation resulting from 5-HT activation of 5-HT$_{1\alpha Pan}$ even when low levels (10^{-5}M (Figure 2-8B) or 5×10^{-8} (not shown)) of 5-HT were used. Several antagonists (clozapine, methiothepin, S(-)propanolol, metergoline, cyproheptadine, SCH23390, S(-)eticlopride) had significant effects on non-induced 5-HT$_{1\alpha Pan}$ cells and could therefore not be tested for activity in induced 5-HT$_{1\alpha Pan}$ cells. Similarly, none of 14 putative antagonists (10μM) were able to block 5-HT (10μM) activation of 5-HT$_{1\alpha Pro}$ (Figure 2-8C, Table 2-3). Because of background activity at endogenous receptors in the non-induced cell line, we were not able to test the 14 other putative antagonists at cells induced to express 5-HT$_{1\alpha Pro}$ (Table 2-3).

Crustacean 5-HT$_{1\alpha}$ receptors therefore appear to be highly resistant to antagonists for unclear reasons. Even the potent and selective mammalian 5-HT$_{1A}$ blocker WAY100635 (Hoyer et al., 2002) was ineffective at 5-HT$_{1\alpha Pan}$. Prazosin, which functionally blocks 5-HT$_{1Dro}$ (Saudou, 1992) also had no effect on 5-HT$_{1\alpha Pan}$. Many of these putative antagonists block functional activation of mammalian (Hoyer et al., 2002) and invertebrate (Witz et al., 1990; Saudou et al., 1992; Li et al., 1995; Barbas et al., 2002; Hobson et al., 2003) 5-HT receptors. While some of these may act as allosteric modulators and therefore be sensitive to the high variability of the N-terminal tail amongst 5-HT receptors (May et al., 2004), several of the drugs do efficiently displace radioligands at arthropod receptors (Tierney, 2001) and therefore presumably bind at or near the ligand binding pocket. For none of these to be capable of blocking 5-HT activation of 5-HT$_{1\alpha Pan}$ is unexpected. 5-HT$_{1\alpha Pan}$ may have an extraordinarily high affinity

and selectivity for 5-HT such that the antagonists are not able to overcome the binding of and/or conformational changes elicited by 5-HT. Radioligand studies done on other arthropod 5-HT$_{1\alpha}$ receptors, however, do not fully support this hypothesis as some putative antagonist drugs bind more strongly than 5-HT (Saudou et al., 1992; Colas et al., 1995). Alternatively, the exogenously expressed 5-HT$_{1\alpha Pan}$ may be in a hyperactive state that is highly susceptible to activation, overcoming any antagonist effects. This could also explain the high response to almost all the putative agonists we tested.

Several putative antagonists appeared to increased the efficacy of the 5-HT effect on cAMP accumulation in cells expressing 5-HT$_{1\alpha Pan}$ or 5-HT$_{1\alpha Pro}$ (Figure 2-8B,C). When tested without 5-HT, however, these drugs had no significant effect on forskolin-stimulated cAMP production in the absence of 5-HT (not shown) indicating that they are not acting as agonists. Further studies will be necessary to determine the relationship between antagonist drugs and the crustacean 5-HT$_{1\alpha}$ receptors.

Although we could not find effective 5-HT$_{1\alpha Pro}$ antagonists, we were able to identify several drugs that would block 5-HT$_{2\beta}$ while not affecting 5-HT activation of 5-HT$_{1\alpha}$ (Table 2-3). Cinanserin, (+)butaclamol and ritanserin all efficiently blocked 5-HT$_{2\beta Pan}$ but had no activity at 5-HT$_{1\alpha Pan}$. Similarly, methiothepin and cinanserin blocked 5-HT$_{2\beta Pro}$ but not 5-HT$_{1\alpha Pro}$. All four of these are effective 5-HT$_{2\beta}$ antagonists but some could not be tested at the 5-HT$_{1\alpha}$ receptor from one or the other species to confirm specificity. To our knowledge, cinanserin has not been tested at other arthropod 5-HT receptors but methiothepin binds relatively strongly to the 5-HT$_{2\alpha Dro}$ receptor (Colas et al., 1995). Methiothepin may therefore be a 5-HT$_2$-specific antagonist in arthropods. Several 5-HT$_1$ and 5-HT$_2$ type receptors from mollusks and nematodes, however, also bind methiothepin (Sugamori et al., 1993; Olde and McCombie, 1997; Angers et al., 1998; Hamdan et al., 1999). Similarly, (+)butaclamol binds a variety of 5-HT receptor subtypes from diverse species (Saudou et al., 1992; Olde and McCombie, 1997; Hamdan et al.,

1999). The antagonist profile of 5-HT receptors therefore appears to be well conserved among crustaceans but this may not extend between phyla.

Discussion

Serotonin has multiple complex effects even in relatively simple crustacean nervous systems. Specific circuits within these systems have been extensively developed as models of modulatory mechanisms and have shown complex cellular and behavioral responses to 5-HT mediated by several putative 5-HT receptors. An understanding of the mechanisms involved in maintaining and restructuring invertebrate 5-HT circuits may aid in elucidating similar processes in more complex systems. While many studies have elucidated the effects of serotonergic modulation, the molecular mechanisms underlying this modulation have been inaccessible due to the scarcity of knowledge about the 5-HT receptors mediating these signals in invertebrates. Because crustacean 5-HT receptors have only recently been identified (Clark et al., 2004; Sosa et al., 2004), interpretation of experiments with various pharmacological agents characterized at vertebrate receptors has been relatively limited. Here we show that two 5-HT receptors are highly conserved in terms of sequence, signaling and pharmacological profiles between two species of crustaceans, *Panulirus interruptus* and *Procambarus clarkii*, belonging to different infraorders. The pharmacological profiles of lobster and crayfish receptors are remarkably conserved, indicating that the drugs characterized here could be used with relative certainty in related crustacean systems. However, not all drugs could be tested on all orthologs of the receptors so care should still be taken when applying these drugs to investigate 5-HT function in physiological preparations, even in related species. In addition, at least three crustacean 5-HT receptors remain uncharacterized (Tierney, 2001; Clark et al., 2004) and may respond to the drugs shown here to be selective for 5-$HT_{2\beta}$ or 5-$HT_{1\alpha}$. The use of strategic combinations of multiple agonists and antagonists

will therefore be necessary to confirm the roles of individual receptors in mediating 5-HT effects in crustacean systems. Future studies aimed towards identification and characterization of the remaining crustacean aminergic receptors will eliminate some of these uncertainties when working in physiological preparations.

Using an antibody directed specifically to crustacean 5-HT$_{1\alpha}$, we previously showed that this receptor was extensively expressed in similar patterns in thoracic ganglia of crayfish and *Macrobrachium rosenbergii*, the giant freshwater prawn (Sosa et al., 2004; Spitzer et al., 2005). Crustacean 5-HT receptors may therefore be conserved in their expression patterns as well as in their molecular structure and function. In crayfish, 5-HT$_{1\alpha}$ is observed in numerous somata and in neuropil throughout the central nerve cord. It is also localized to processes surrounding the nerve roots, to superficial flexor muscles of the abdomen and to processes on the vasculature. In addition, while the same structures in the nerve cord label for 5-HT$_{1\alpha}$ in all animals, expression levels of this receptor are highly variable between individuals (Spitzer et al., 2005, Chapter 4). The 5-HT$_{1\alpha}$ receptor therefore provides numerous targets for 5-HT modulation which may change in an experience- or state-dependent manner in the crayfish.

Several of the drugs characterized here have been used previously to investigate physiological mechanisms of 5-HT neuromodulation in crustaceans. Based on the pharmacological characterization of crustacean 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ we can now hypothesize which receptors might be involved in mediating specific effects and behaviors. In the lateral giant escape system of crayfish, the agonist mCPP was able to elicit the inhibitory component of the 5-HT response, regardless of social status (Yeh et al., 1997) indicating that inhibition may be mediated by the 5-HT$_{1\alpha Pro}$ receptor. The facilitatory component of the 5-HT response was elicited by α-Me-5-HT (Yeh et al., 1997) which we found to be an effective agonist of both 5-HT$_{2\beta Pro}$ and 5-HT$_{1\alpha Pro}$. One or both of these receptors may therefore contribute to facilitation of the

circuit. Pharmacological and/or expression studies will be necessary to identify the responsible receptor(s). When injected into freely behaving crayfish, mCPP closely mimicked the effects of 5-HT on posture (Tierney and Mangiamele, 2001; Tierney et al., 2004) indicating that $5\text{-HT}_{1\alpha Pro}$ may be important in transducing 5-HT modulation of posture. Crayfish abdominal muscles, essential to posture, are modulated by 5-HT at the level of the motor neurons and at the neuromuscular junction (Strawn et al., 2000). $5\text{-HT}_{1\alpha Pro}$ receptors are localized on the abdominal superficial flexor motorneurons (Spitzer et al., 2005) and may therefore mediate this effect. In addition, 5-CT enhances agonistic behavior in *Procambarus* (Tierney and Mangiamele, 2001), indicating that $5\text{-HT}_{2\beta Pro}$ and/or $5\text{-HT}_{1\alpha Pro}$ may mediate 5-HT signals eliciting these behaviors. Because posture is an essential component of agonistic behavior, these receptors may therefore contribute to different aspects of the agonistic behavioral program. The role of 5-HT in agonistic interactions between crayfish has been intensely studied (Edwards and Kravitz, 1997; Huber et al., 1997b; Kravitz, 2000) and the localization of $5\text{-HT}_{2\beta Pan}$ in the nervous system in addition to $5\text{-HT}_{1\alpha Pro}$ localization (Spitzer et al., 2005) may facilitate understanding of the mechanisms underlying this complex group of behaviors. Such a study could also direct further investigations into the pharmacological or molecular properties of specific circuits. Although $5\text{-HT}_{1\alpha Pro}$ is expressed by neurons throughout the crayfish eyestalk (Spitzer et al., 2005), it does not appear to be responsible for mediating the neurohormonal 5-HT signal that results in increases in hemolymph glucose levels as this was not mimicked by mCPP (Lee et al., 2000). $5\text{-HT}_{2\beta Pro}$ may, however, be involved as the response was evoked by both 5-CT and 8-OH-DPAT. In the stomatogastric nervous system of the crab, *Cancer borealis*, cinanserin blocked 5-HT-mediated acceleration of the pyloric rhythm (Zhang and Harris-Warrick, 1994) suggesting that the crab $5\text{-HT}_{2\beta}$ ortholog may play a role in modulation of pyloric frequency.

As we demonstrate in comparing crustacean $5\text{-HT}_{2\beta}$ and $5\text{-HT}_{1\alpha}$ receptor

pharmacology, many drugs are active at more than one 5-HT receptor from the same species. Interpretations of studies utilizing individual drugs must therefore be taken with the understanding that uncharacterized 5-HT receptors could also be contributing to observed effects. Especially when using a single agonist drug to elicit a '5-HT effect', the possibility of activating multiple 5-HT and other aminergic receptors must be kept in mind. In addition, radioligand displacement assays of the *Drosophila* 5-HT$_{1\alpha}$ ortholog and its paralog, 5-HT$_{1\beta Dro}$, indicated that arthropod 5-HT receptor paralogs can have very similar pharmacological properties (Saudou et al., 1992) and their effects may therefore be difficult to tease apart even using combinations of drugs. Profiling of additional receptor subtypes, the use of multiple characterized drugs and expression studies should allow greater confidence in assigning specific receptor types to behavioral effects.

In addition to developing a toolset for crustacean researchers, we have found that pharmacological function is well conserved between distantly related infraorders of decapod crustaceans. A significant portion of the 5-HT$_{1\alpha}$ receptor from the giant freshwater prawn, *Macrobrachium rosenbergii*, has been cloned and is also highly conserved with its lobster and crayfish orthologs (Sosa et al., 2004). This suggests that the conservation of pharmacological function observed between the species studied here may extend beyond reptantian crustaceans which might allow the use of these drugs to investigate 5-HT mechanisms in diverse crustacean systems. Other arthropod 5-HT$_1$ and 5-HT$_2$ receptors have been characterized pharmacologically using binding assays (Saudou et al., 1992; Colas et al., 1995). Because binding of a drug to a receptor does not necessarily indicate functionality, comparisons of these data must be made carefully. Several 5-HT receptors from more distantly related invertebrates such as mollusks (Li et al., 1995) and nematodes (Hamdan et al., 1999; Hobson et al., 2003) have been pharmacologically characterized using functional assays. However, while signaling mechanisms of these receptors is conserved with their arthropod orthologs, their

pharmacological properties are not (Tierney, 2001).

In summary, by expressing cloned crustacean 5-HT receptors in cell lines and using functional assays of receptor activation, we have identified several antagonists, (+)butaclamol, cinanserin and ritanserin for *Panulirus* and methiothepin and cinanserin for *Procambarus*, that will block 5-HT$_{2\beta}$ while leaving 5-HT$_{1\alpha}$ unaffected. In addition, mCPP weakly activates 5-HT$_{1\alpha}$ while having no effect on 5-HT$_{2\beta}$ for both species while quipazine activates 5-HT$_{1\alpha Pro}$ but not 5-HT$_{2\beta Pro}$. Combinations of these drugs can be applied in studies that examine the mechanisms underlying 5-HT modulation in identified circuits and cells of lobster, crayfish and related crustacean nervous systems.

Chapter 3 – The role of 5-HT$_{2\beta Pan}$ and 5-HT$_{1\alpha Pan}$ in serotonergic modulation of the pyloric rhythm.

Introduction

　　Rhythmic motor patterns such as locomotion, respiration and chewing are essential to the function and survival of an animal. The central pattern generators responsible for producing such rhythmic outputs depend on neuromodulation to maintain a rhythmic cycle and to appropriately modify their output parameters (Thoby-Brisson and Simmers, 1998; Marder and Bucher, 2001). Neuromodulation can function to extend the performance range of a circuit by orchestrating state dependent reconfiguration of circuits and intrinsic properties of component cells on various time scales. Within these different states the same modulator can have different effects through the actions of multiple receptors or through changed signaling pathways. As a result, complex circuits may therefore experience state-dependent variability at multiple levels and on different time scales. Several relatively simple invertebrate circuits such as the stomatogastric nervous system (STNS) of decapod crustaceans, which has been extensively characterized, are therefore ideal subjects for dissecting the mechanisms underlying state-dependent effects in neuromodulatory systems.

　　The STNS of crustaceans is a well-established model for investigating the circuitry and neuromodulation of motor pattern generation. The STNS contains multiple small, defined circuits, each of which drives a different set of muscles to produce patterned activity associated with specific functions. One such circuit, the pyloric network, consists of 14 identified neurons that fall into 6 cell types in the spiny lobster, *Panulirus interruptus* (Selverston et al., 1976). The intrinsic firing properties and synaptic connectivities of each cell type have been described in great detail (Harris-Warrick et al., 1992b; Nusbaum and Beenhakker, 2002; Selverston, 2005). In addition,

the effects of various neuromodulators including serotonin (5-HT) on pyloric motor output have been investigated at the cellular and circuit levels (Beltz et al., 1984; Flamm and Harris-Warrick, 1986a, b; Katz and Harris-Warrick, 1990; Harris-Warrick et al., 1992a; Harris-Warrick et al., 1998; Ayali and Harris-Warrick, 1999; Peck et al., 2001).

5-HT alters the firing properties of isolated pyloric neurons in a cell-specific manner. In *Panulirus*, the pyloric pacemaker neuron, AB (anterior burster), oscillates faster in 5-HT while the VD (ventricular dilator) and LP (lateral pyloric) neurons are simultaneously inhibited. The IC neuron is excited by 5-HT and there is no effect of 5-HT on isolated PD (pyloric dilator) or PY (pyloric) neurons (Flamm and Harris-Warrick, 1986b; Ayali and Harris-Warrick, 1999). In addition to effects on intrinsic properties of pyloric cells, 5-HT also modulates the strength of electrotonic and chemical synapses within the circuit including graded neurotransmitter release (Johnson and Harris-Warrick, 1990; Johnson et al., 1994, 1995). This modulation is significant enough to reverse the sign of some net synaptic interactions (Johnson et al., 1994). In some instances 5-HT is known to alter specific ionic currents in a cell-specific manner (Kiehn and Harris-Warrick, 1992; Zhang and Harris-Warrick, 1995; Harris-Warrick et al., 1998; Peck et al., 2001) and different amines can elicit pyloric bursting via multiple ionic mechanisms in the isolated AB neuron (Harris-Warrick and Flamm, 1987).

The unique response to 5-HT of each pyloric cell type could arise from cell-specific expression patterns of different types of 5-HT receptors (5-HTRs), differential activation of signaling cascades by the same 5-HTRs or through altered downstream targets, such as ion channels (Baro et al., 1994; Baro et al., 1997; Baro and Harris-Warrick, 1998; Baro et al., 2000; Mizrahi et al., 2001). Arthropods are known to express multiple 5-HTR types (Tierney, 2001). A total of 5 arthropod 5-HT receptor subtypes have been cloned and characterized to date (Witz et al., 1990; Saudou et al., 1992; Colas et al., 1995; Clark et al., 2004). In addition, analysis of arthropod genomic databases

suggest the presence of three putative monoamine receptors that currently remain uncharacterized (Clark et al., 2004). Arthropods could therefore express a total of up to eight 5-HTR types.

Neuromodulators such as 5-HT are critical to the initiation, maintenance and plasticity of CPG circuits. In order to understand how these substances exert their effects, the mechanisms mediating their actions within neurons of the circuit must be known. While the cellular effects and final targets of the modulators have been studied in detail, little is known about the transduction cascades that underlie these effects in identified cells of a CPG. Previously we cloned and characterized 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ receptors from *Panulirus* (Clark et al., 2004; Sosa et al., 2004) (Chapter 2). Here we apply these newly identified pharmacological tools to investigate the role of each receptor in 5-HT modulation of the pyloric rhythm.

Methods and Materials

Animals

Lobsters, *Panulirus interruptus,* were obtained from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained at 16ºC in continually filtered and aerated artificial seawater. Animals were fed once per week with raw shrimp (Kroger, Atlanta). The number of shrimp added to the tank was 2-5 more than the number of lobsters in the tank, but no attempt was made to ensure that each lobster ate.

Electrophysiological recordings

Lobsters were anesthetized for at least 30min on ice after which the stomatogastric nervous system was dissected out and pinned in a Sylgard-lined Petri dish using standard techniques (Selverston et al., 1976). The stomatogastric ganglion (STG) and a portion of the stomatogastric nerve (*stn*) were desheathed. The preparation

was bathed in *Panulirus* saline consisting of (in mM) 479 NaCl, 12.8 KCl, 13.7 $CaCl_2$, 39 Na_2SO_4, 10 $MgSO_4$, 2 glucose, 4.99 HEPES, 5 TES at pH 7.4. All experiments were carried out at room temperature. Petroleum jelly (Vaseline) wells (1-2cm across) were built around the STG and around the desheathed portion of the *stn*. Both wells were always tested for leaks with saline containing 0.005% Fast Green. The well around the STG was constantly perfused at 2ml/min with saline or drug solutions and tests with Fast Green prior to every experiment showed that the solution in the STG well was exchanged in less than 20s.

Extracellular recordings from the pyloric dilator nerve (*pdn)*, medial ventricular nerve (*mvn)* and lateral ventricular nerve (*lvn)* were obtained with stainless steel pin electrodes, isolated with Vaseline, and a differential AC amplifier (A-M Systems, Everett, WA). In some preparations, the activity of the pyloric dilator (PD) neuron was monitored with intracellular somatic recordings using glass microelectrodes filled with 3M KCl (20-30 $M\Omega$) and an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). In these cases, the PD was identified by the characteristic shape of its oscillation, by its timing in the motor pattern and by a 1:1 correlation between action potentials recorded intracellularly from the soma and extracellularly from the *pdn*.

After recording baseline activity for 10min, the saline solution in the *stn* well was exchanged for 1M sucrose to block descending neuromodulatory input. Fast Green was included in the sucrose solution to detect leakage during the experiment. After one hour in the sucrose block, experiments to determine the effects of 5-HT receptor agonists and antagonists were performed. For experiments to determine the effect of the agonist mCPP, 10μM 5-HT was applied for 5min and washed out for 1hr, followed by a 5min application of 100μM mCPP which was then washed out for 1hr. The antagonist (+)butaclamol (10μM) was then applied for 5min followed immediately by a 5min application of (+)butaclamol + mCPP. After this regime, the experiment was terminated

and the preparation was not used to test additional drugs. In some experiments, the order of 5-HT and mCPP application were reversed. No difference was observed. For experiments involving antagonists (cinanserin and (+)butaclamol), a sucrose block was applied after recording 10min of baseline. An hour later 5-HT was applied for 5min and washed out for 1hr. The antagonist was then applied alone for 5min and then in combination with 5-HT for 5min followed by a 1hr wash. After application of one antagonist the experiment was terminated and the preparation was not used further.

Electrophysiological data acquisition and analysis.

Data were acquired using a Digidata 1322A data acquisition board (Axon Instruments) and Axoscope software. The data were subsequently analyzed with DataView4.7a (W.J. Heitler, University of St. Andrews, Scotland) and Microsoft Excel. We measured agonist- and antagonist-induced changes in 2 parameters: pyloric cycle frequency and spikes per burst of the VD (ventricular dilator) neuron. To analyze changes in cycle frequency, we used threshold analysis from extracellular (*pdn*) or intracellular recordings of PD activity. One cycle was defined as the period extending from the first spike in one PD neuron burst to the first spike in the following PD burst. VD spike detection from extracellular *mvn* recordings was performed either by voltage thresholding or by using a spike shape template function.

Cycle frequency and VD spikes per cycle data were first plotted as a function of time for each experiment. Baseline spike and cycle frequencies were then determined from ten cycles just before application of a drug during a period when the preparation was in a stable cycling state (i.e. one hour after the sucrose block was applied or at the end of a one hour wash period). The peak response to 5-HT, mCPP, antagonist and antagonist + 5-HT was identified from the time course plots during each 5 minute drug application. Cycle and VD spike frequencies were determined from 10 cycles during this

peak effect. In some preparations (3/18) 5-HT application almost completely eliminated the pyloric cycle and 10 cycles could not be observed during the peak 5-HT effect. In these cases, we obtained cycle frequency by counting the number of cycles in a 30 second period during the peak, and dividing that number by 30. Within an experiment the time course of the peak 5-HT response correlated well with the time course of the peak 5-HT + antagonist response. Statistical significance for physiology data was determined using Student's paired t-tests in Excel and indicated when $p<0.05$. Bar graphs were constructed in Prism (GraphPad) and all final figures were composed in Adobe Illustrator CS.

Results

The pyloric cycle exists in two distinct states with different 5-HT responses

The pyloric circuit in the STNS of *Panulirus* consists of 6 cell types interconnected by electrical and inhibitory chemical synapses (Figure 3-1A). The circuit is driven by a pacemaker ensemble consisting of an AB pacemaker interneuron that is electrically coupled to two PD motorneurons. In addition, two follower neurons, VD and LP, feed back onto the pacemaker kernel and play an important role in governing cycle frequency (Weaver and Hooper, 2003). Endogenous oscillations in the AB neuron and consequent activity of coupled and follower neurons give rise to a stereotyped motor output that can be measured extracellularly on efferent motor nerves (Figure 3-1B, top). Descending modulatory input can be blocked by placing a pool of sucrose on the single modulatory input nerve (stomatogastric nerve, *stn*). After an hour of sucrose block the pyloric cycle stablilizes in a basal cycling state; the pacemaker kernel cycles weakly, VD firing is reduced, and the LP, PY and IC neurons are silenced (Figure 3-1B, middle).

Once endogenous neuromodulation is removed with the sucrose block, the effect of individual modulators on circuit properties can be assessed. Using this approach in combination with techniques to synaptically isolate neurons, it was previously

Figure 3-1: The pyloric rhythm of the stomatogastric nervous system in spiny lobster exists in two distinct states which are differentially modulated by 5-HT. **A**: Schematic of the pyloric circuit in *P. interruptus* (Based on Ayali and Harris-Warrick, 1999). **B**: Sequential recordings from two preparations before sucrose block (top), in the basal cycling state (middle) and in 10^{-5}M 5-HT (bottom). Depending on the state of the preparation, cyle frequency was either accelerated by 5-HT (left) or slowed by 5-HT (right). Extracellular recordings from the *pdn* (upper) and *mvn* (lower) are shown. **C**: Two example timecourse plots of cycle frequency for both types of preparations (accelerated, left; slowed, right). Each point marks a single pyloric burst.

A

B

5-HT - increase   5-HT - decrease

Intact

pdn
mvn

IC   VD   VD

Sucrose block

pdn
mvn

VD   VD

5-HT

pdn
mvn

IC

1 sec

C

5-HT - increase   5-HT - decrease
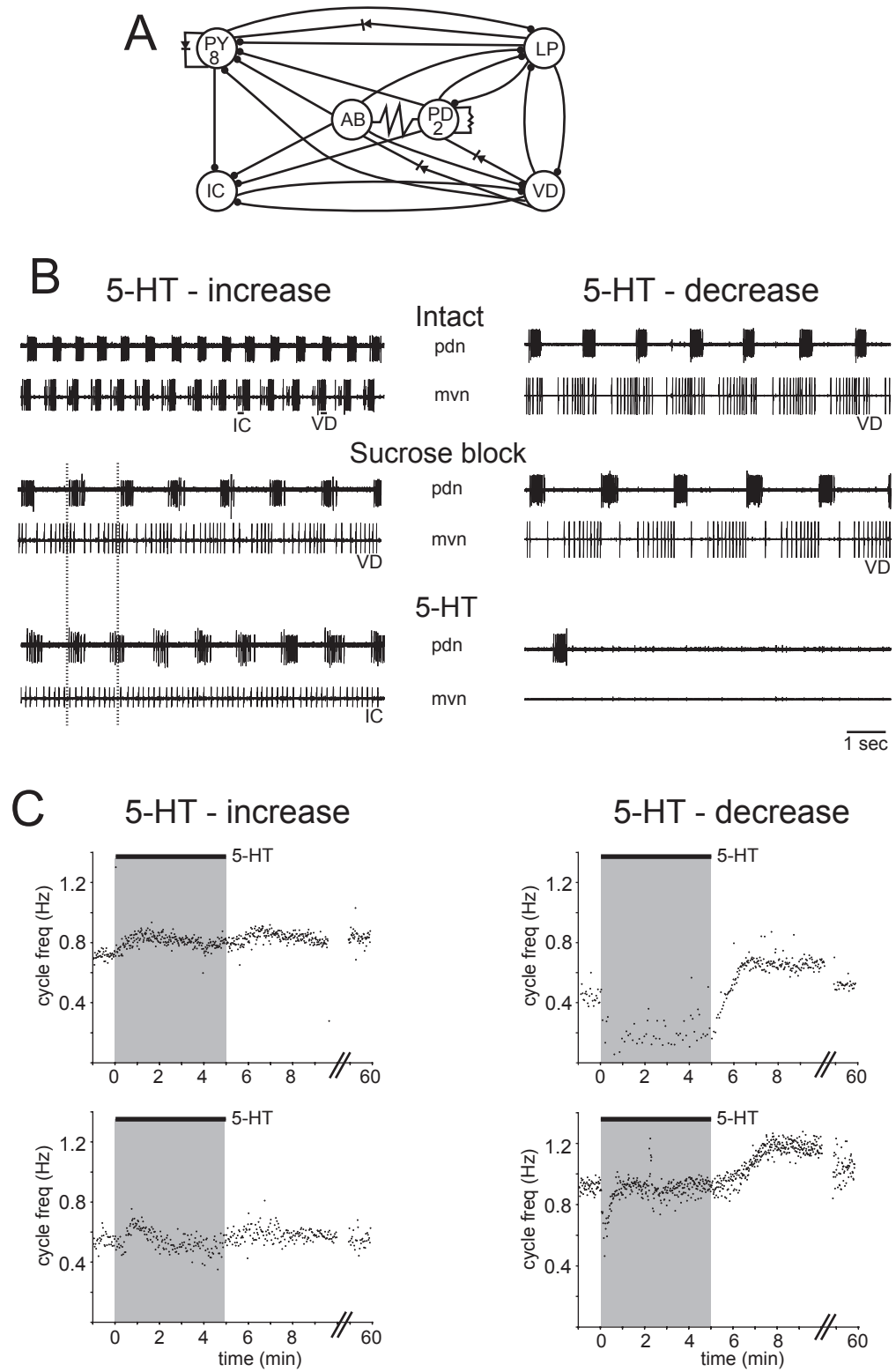
demonstrated that in *Panulirus interruptus*, 5-HT acts on the AB to excite the pacemaker neuron and increase cycle frequency (Flamm and Harris-Warrick, 1986a, b; Ayali and Harris-Warrick, 1999). 5-HT also excited IC, inhibited LP and VD and had no effect on PD and PY (Flamm and Harris-Warrick, 1986b). The AB, PD, LP and VD neurons are key regulators of cycle frequency (Miller and Selverston, 1982; Beltz et al., 1984; Ayali and Harris-Warrick, 1999; Weaver and Hooper, 2003), and 5-HT directly alters the activity of three of these cells: AB, LP and VD (Flamm and Harris-Warrick, 1986b). The directionality of this modulation differs depending on cell type suggesting that multiple 5-HT receptors might be involved in mediating 5-HT modulation.

We therefore chose to study the role of the $5\text{-HT}_{2\beta\text{Pan}}$ and $5\text{-HT}_{1\alpha\text{Pan}}$ receptors in modulating cycle frequency. After modulatory input was removed with a sucrose block, the LP was silent in 16 of 18 preparations. In the remaining 2 preparations, LP fired very weakly and irregularly. We reasoned that further 5-HT inhibition of LP would minimally affect cycle frequency, and therefore we did not analyze LP activity in this work. We monitored VD with extracellular electrodes on the *mvn*. Since AB is electrotonically coupled to PD (and they fire synchronously), and 5-HT has no effect on the isolated PD (Flamm and Harris-Warrick, 1986b; Ayali and Harris-Warrick, 1999), we monitored 5-HT's effects on the AB neuron with intracellular and/or extracellular (*pdn*) recordings of PD.

Figure 3-1B shows serial recordings of *pdn* and *mvn* activity from two representative preparations before sucrose block (top), one hour after sucrose block (middle) and at the peak of the 5-HT effect (bottom). We found that in 7 of 18 preparations pyloric cycle frequency was initially enhanced (left), while in 11 preparations cycle frequency was initially reduced (right) by 10μM 5-HT application (*pdn* traces). The difference in response did not correlate with differences in sex, molt stage or the presence/absence of a sperm pac. In all preparations, spiking in VD was

inhibited by 5-HT (Figure 3-1B, bottom, *mvn* trace) and this inhibition was sustained throughout the 5min 5-HT application.

For each experiment time course graphs were obtained by plotting the frequency of each pyloric cycle over the time before, during and after 5-HT application (Figure 3-1C). Two examples of time courses are shown for each of the 5-HT-induced responses in cycle frequency. Among preparations, the time course profile of the 5-HT effect on cycle frequency was variable but in most cases followed a biphasic profile. In two instances 5-HT-induced increases in cycle frequency were observed to be triphasic and in one preparation the cycle frequency slowly rose so that a stable peak was not attained before commencement of the wash. The 5-HT effects appeared to operate on several distinct time scales. The peak effect occurred immediately upon 5-HT entry to the bath in 91% preparations where 5-HT application slowed cycle frequency while the peak effect in accelerated preparations occurred 20-30s later. The response during the remainder of the 5min 5-HT application was variable for both directions of response. The complexity and variability of the 5-HT response time course indicate that these effects may involve several 5-HT receptors and/or multiple cellular targets.

All 5-HT effects were reversible and visibly washed out within minutes after 5-HT removal. In 60% of preparations, cycle frequency increased within the first five minutes of wash initiation (see Figure 3-1C for example). 5-HT receptors in the STG may therefore be configured to detect changes in 5-HT levels as opposed to simply responding to 5-HT signals in an on/off fashion. Indeed, *Panulirus* pyloric cells are exposed only to neurohormonal 5-HT and respond to nM levels of the amine (Beltz et al., 1984). More studies will, however, be required to address the question of bidirectional sensitivity. After one hour of washing, cycle frequency was significantly increased in 15 of 18 preparations; this may be due to a slow rebound effect related to 5-HT application or to drift in the preparation over time. In several (n=5) experiments we reapplied 5-HT

after the one hour wash. The 5-HT effect was always the same in direction and amplitude as observed during the first trial (n=2 for increase, n=3 for decrease, p=0.4).

The magnitude of change in cycle frequency in peak response to 5-HT varied between preparations and did not correlate with the cycle frequency of the intact preparations before sucrose block (Figure 3-2A). We therefore divided our preparations into two groups. Class I preparations were defined as those in which 5-HT causes an increase in cycle frequency from the sucrose blocked state while in Class II preparations 5-HT slows cycle frequency. Interestingly, while the initial cycling frequencies of Class I and Class II preparations do overlap, Class I preparations started with a significantly faster mean cycle frequency in the intact preparation (1.25$\pm$0.09 vs. 0.89$\pm$0.08, p<0.02) (Figure 3-2B). After one hour of sucrose block to remove modulatory input, however, cycle frequencies of Class I and Class II preparations are indistinguishable. These data suggest that there is a stable intrinsic difference in the pyloric circuit between the Class I and Class II responders, which is uncovered only when descending neuromodulation is present.

For each experiment, the peak effect of 10μM 5-HT on cycle frequency and VD spikes per cycle was measured from time course plots such as those in Figure 3-1C. The average percent change from baseline in cycle frequency and VD activity during 10μM 5-HT application (measurements immediately before 5-HT application but after the one hour sucrose block) for all preparations is summarized in Figure 3-2C. Cycle frequency was increased by an average of 29$\pm$3% in Class I preparations and reduced by 58$\pm$12% in Class II preparations (Figure 3-2C, top). On average, the VD was inhibited by 98$\pm$2% from baseline throughout all 5-HT applications (Figure 3-2C, bottom).

Our findings are consistent with those previously published by Beltz et al. (1984) who reported that 5-HT effects varied with the preparation. In their studies, 5-HT increased or decreased pyloric cycle frequency in *P. interruptus* in ~33% and ~67% of the
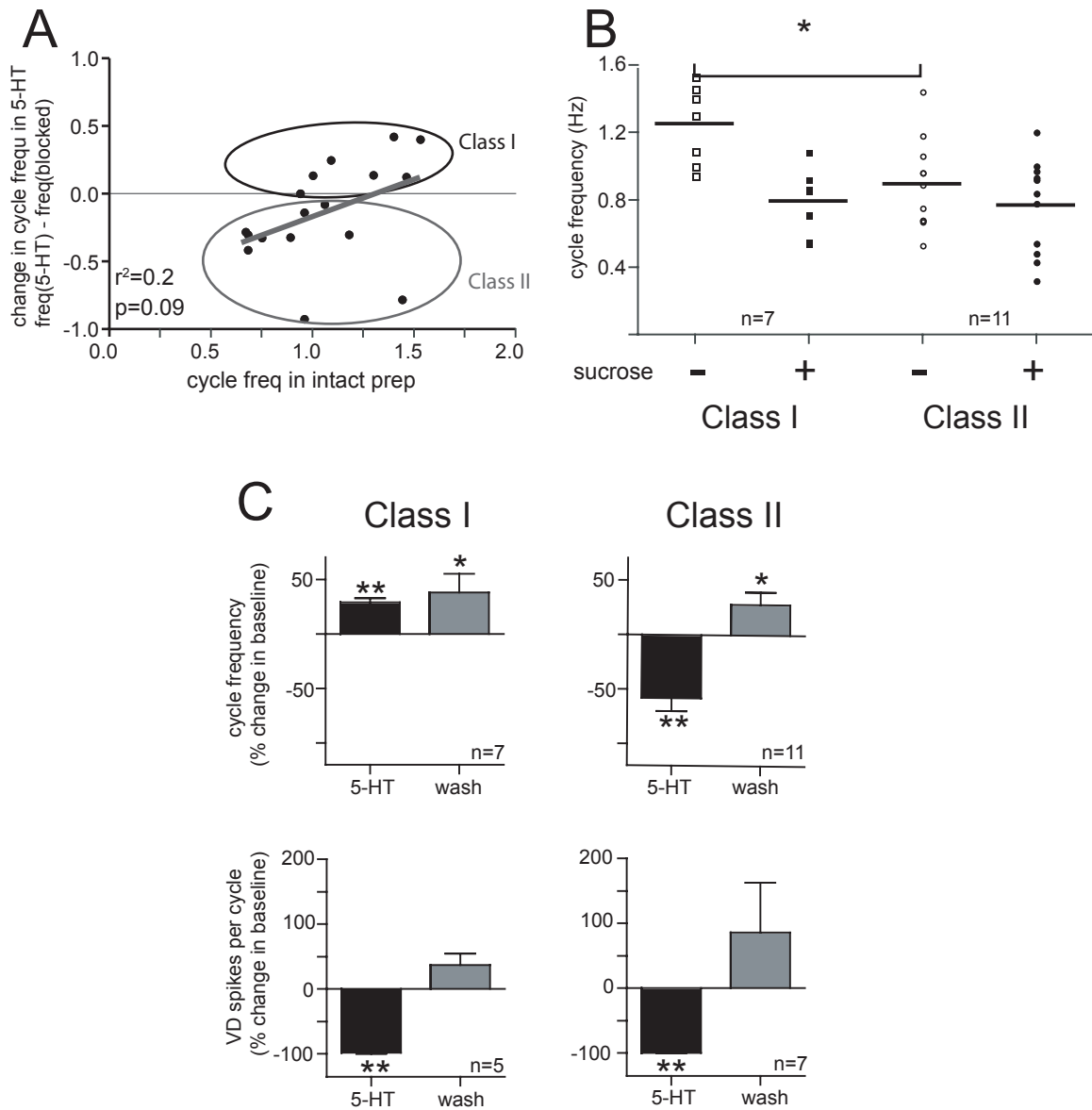
Figure 3-2: 5-HT has different effects on two classes of STNS preparation. State corre-lates with cycle frequency in the intact preparation. **A**: Change in cycle frequency as a result of application of 10^{-5}M 5-HT as a function of cycle frequency in the intact prepara-tion. Each point represents a single preparation. The 5-HT response is slightly correlated with the cycle frequency of the intact preparation. The population can be separated into Class I and Class II based on the direction of the 5-HT response. **B**: The average cycle frequency of the intact preparation in class I (open squares) is significantly higher than that of class II (open circles). *p< 0.02. However, all preparations cycle at the same speed after sucrose block (filled symbols). **C**: Summary of 5-HT effects on pyloric cycle frequency (top) and VD spike activity (bottom) in class I (left) and class II (right) prepa-rations. **p<0.001, *p<0.05.

preparations, respectively. On the other hand, a Class I response was always observed in studies from the Harris-Warrick lab (Flamm and Harris-Warrick, 1986a; Ayali and Harris-Warrick, 1999). The reason for this discrepancy across laboratories is not clear. It was previously suggested that differences in LP activity could account for these observations (Beltz et al., 1984; Flamm and Harris-Warrick, 1986a), but in our experiments LP was silent in 16 preparations. In the two preparations where LP fired weakly one was a Class I responder and the other a Class II. Alternatively, there are obvious differences in the experimental protocols. Our studies are conducted at room temperature, while experiments in the Harris-Warrick lab are conducted at 16°C; however, Beltz et al. (1984) conducted their experiments at 10-14°C. The response to 5-HT is known to vary with the application protocol (Teshiba et al., 2001) and our perfusion system was slower than previously reported (2ml/min vs. 5-12ml/min). Additionally, feeding alters 5-HT levels in specific neurons (B. Musolf, unpublished) and 5-HT levels can regulate receptor expression (Sanders-Bush, 1990; Wohlpart and Molinoff, 1998a). Perhaps feeding regimes or the length of time in captivity vary across labs. Environmental differences such as saltwater supply or light:dark cycles could also exist between labs. At present, the reason for the different findings across labs remains intriguing but elusive.

Many phenomena affecting one or more components of the 5-HT signal transduction cascade could underlie these state-dependent differences. Proteins involved anywhere in the signaling cascade ranging from receptor to ion channel might vary at the level of gene expression. On the other hand, gene expression may be identical in all cases, but state-dependent changes could alter cross-talk between the signaling cascades (Werry et al., 2005) or receptor coupling (Hamm, 1998; Heuss and Gerber, 2000; Brzostowski and Kimmel, 2001) and hence, the 5-HT elicited response. To determine if and how $5\text{-HT}_{2\beta\text{Pan}}$ and $5\text{-HT}_{1\alpha\text{Pan}}$ receptor function contributes to the 5-HT effects and differences between the classes, we applied pharmacological tools developed for

Panulirus 5-HT receptors (Chapter 2) that allowed us to investigate the roles of these two receptors in the Class I and Class II 5-HT responses.

5-HT$_{2\beta Pan}$ mediates the 5-HT-induced increase in cycle frequency but not the decrease in VD firing in Class I responders

We used the antagonists (+)butaclamol and cinanserin to investigate the role of the 5-HT$_{2\beta Pan}$ receptor in 5-HT modulation of pyloric cycle frequency. These drugs effectively block 5-HT activation of 5-HT$_{2\beta Pan}$ but not 5-HT$_{1\alpha Pan}$ (Chapter 2). In these serial experiments modulatory input was blocked with sucrose on the *stn* and the preparation stabilized in a basal cycling state for at least an hour. Baseline measurements of cycle frequency and VD spikes per cycle were measured immediately before application of 10μM 5-HT followed by a one hour wash. Measurements for 5-HT and wash were normalized to baseline. A new baseline was measured immediately before application of antagonist because of the observed increase in baseline that occurred during wash in most preparations. Antagonist (10μM) was applied alone for 5min and then together with 10μM 5-HT followed by a one hour wash. The antagonist, antagonist plus 5-HT and wash measurements are normalized to the baseline taken immediately before antagonist application. A time course plot of pyloric cycle frequency for a representative experiment is shown in Figure 3-3A, circled numbers refer to the time points at which measurements were taken for analysis of 5-HT effects with and without antagonist as well as the wash and baseline measurements.

In Class I responders, (+)butaclamol eliminated the increase in cycle frequency in response to 5-HT and unmasked a 5-HT-evoked reduction in cycle frequency. Figure 3-3B shows that 10μM 5-HT alone produced a $33\pm5\%$ mean increase in cycle frequency over baseline ($p < 0.02$) in Class I preparations, while in (+)butaclamol (10μM) the mean cycle frequency was reduced by $27\pm8\%$ ($p < 0.03$) in 5-HT. When 5-HT$_{2\beta Pan}$ was blocked

Figure 3-3: 5-HT accelerates the pyloric rhythm via 5-HT$_{2\beta Pan}$ in class I preparations. This receptor is not involved in VD inhibition by 5-HT. **A**: Time course graph of representative experiment showing pyloric cycle frequency during serial application of 5-HT and antagonist alone and with 5-HT. Serial applications are separated by one hour of wash. Circled numbers correspond to timepoints for measurements presented in bar graphs below. **B**: 10μM 5-HT significantly increases cycle frequency compared to baseline (1). Cycle frequency is increased after a one hour wash following 5-HT application (2). A new baseline was set just before application of 10μM (+)butaclamol, a 5-HT$_{2\beta Pan}$ antagonist which has no effect on cycle frequency alone (3) but blocks the 5-HT effect. In addition, a reduction in cycle frequency in response to 5-HT is unmasked in the presence of butaclamol (4). Cycle frequency is not changed after a one hour wash (5). **C**: Similar results were obtained with a second 5-HT$_{2\beta Pan}$ blocker, cinanserin. **D, E**: Spiking in the VD neuron is completely inhibited in 10μM 5-HT. Neither 5-HT$_{2\beta Pan}$ antagonist succeeds in blocking this effect. # $p<0.05$ vs. baseline; *$p<0.05$ for indicated comparison.
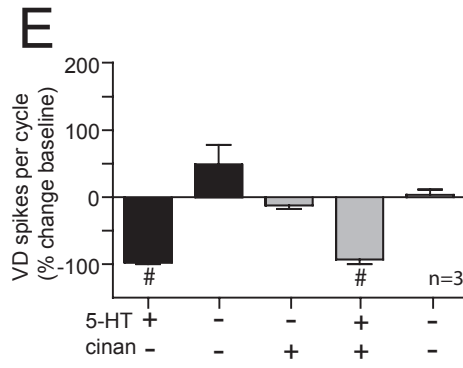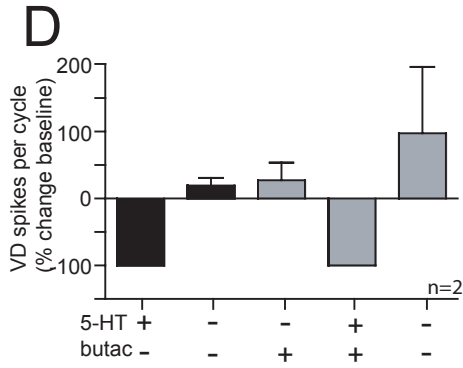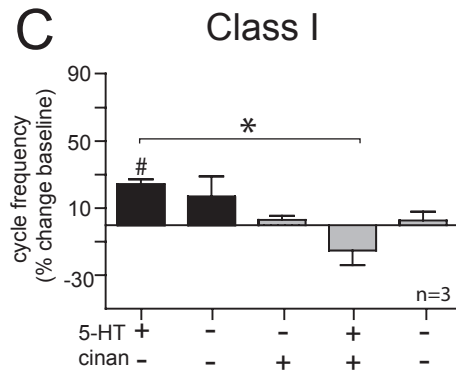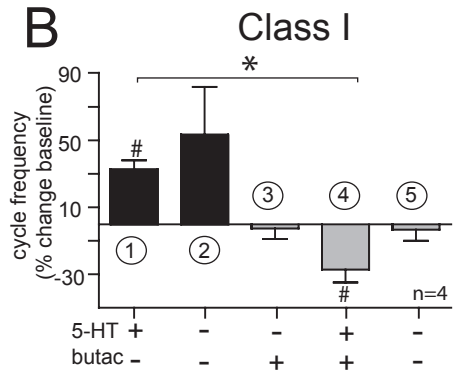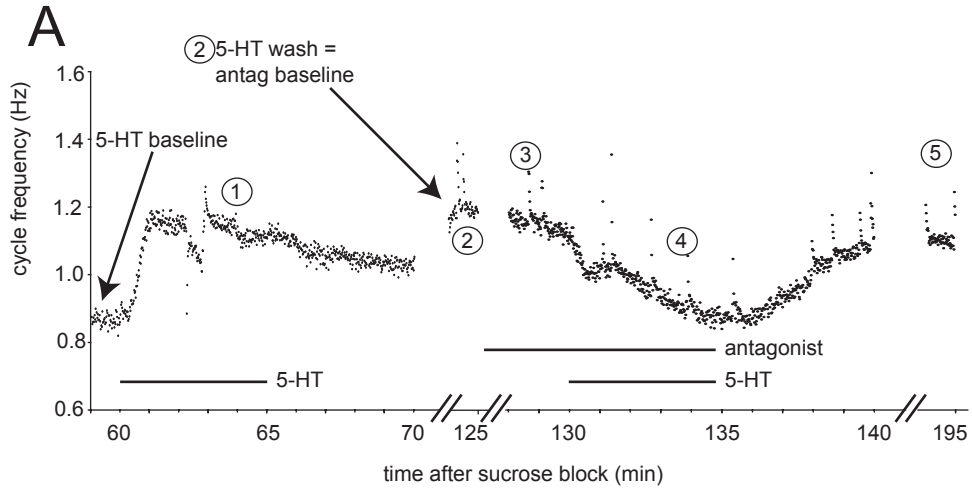
with a second 5-HT$_{2\beta Pan}$ antagonist, cinanserin, the effects were the same. Figure 3-3C shows that in the presence of 5-HT alone, mean cycle frequency was increased by 24$\pm$3% over baseline, but in the presence of 5-HT plus cinanserin, mean cycle frequency was reduced by 15$\pm$9% below baseline. Preliminary data using the antagonist ritanserin, which also blocks 5-HT$_{2\beta Pan}$ and not 5-HT$_{1\alpha Pan}$, resulted in the same responses (not shown) confirming that these effects are most likely mediated by 5-HT$_{2\beta Pan}$. In addition, the time course of the 5-HT response in 5-HT$_{2\beta Pan}$ antagonists was altered. It was no longer variable, but rather sustained and highly stereotyped (see Figure 3-3A).

Neither antagonist had any effect on VD's response to 5-HT. In the presence of (+)butaclamol (Figure 3-3D) or cinanserin (Figure 3-3E) 5-HT still completely inhibited VD firing. The activity of the IC cell is visible on most *mvn* recordings (Figure 3-1B). Like LP, IC was silent after the sucrose block was applied. However, consistent with previous studies (Flamm and Harris-Warrick, 1986a), in some preparations 5-HT excited the IC. The antagonists had no effect on 5-HT-induced excitation of IC in Class I preparations (n=2, data not shown).

Together with previous studies, these data suggest that 5-HT$_{2\beta Pan}$ is expressed exclusively in the AB neuron in Class I responders. Previous work determined that for Class I responders 5-HT acts directly on the AB, LP, VD and IC, but not on the PD or PY neurons (Flamm and Harris-Warrick, 1986b). LP is not active under our experimental conditions and our experiments suggest that 5-HT$_{2\beta Pan}$ receptors do not mediate the VD or IC response to 5-HT; therefore, by process of elimination, 5-HT$_{2\beta Pan}$ receptors responsible for Class I responses are most likely expressed on AB neurites in the STG. Consistent with this idea, 5-HT$_{2\beta Pan}$ receptors are present on fine processes within the STG neuropil, but not in somatic plasma membranes (Clark et al., 2004). Regardless of the antagonist used, blocking of 5-HT$_{2\beta Pan}$ unmasked a reduction in cycle frequency in addition to

removing the frequency increase in response to 5-HT. This inhibition is likely due to removal of the VD neuron from the circuit (see below).

5-HT$_{2\beta Pan}$ contributes to the 5-HT-induced decrease in cycle frequency but not the decrease in VD firing in Class II responders

5-HT$_{2\beta Pan}$ antagonists altered the response of Class II responders to 5-HT such that the 5-HT-induced reduction in cycle frequency was smaller in the presence of antagonists. Figure 3-4A shows that in the presence of 10μM (+)butaclamol, 5-HT (10μM) significantly reduced mean cycle frequency by 28$\pm$8%, as opposed to 53$\pm$16% in the absence of (+)butaclamol. Similarly, 5-HT induced a 34$\pm$20% reduction in mean cycle frequency in the presence of cinanserin and a 75$\pm$25% reduction in the absence of cinanserin (Figure 3-4B). We do not believe that this is due to phenomena such as habituation or receptor desensitization between serial experiments, because the response to repetitive 5-HT applications was not changed in Class II preparations (n=3, p=0.5). In addition, if Class II preparations simply lacked the 5-HT$_{2\beta Pan}$ receptors responsible for the Class I response, the Class II response to 5-HT alone should be comparable to the Class I response in 5-HT$_{2\beta Pan}$ antagonist. Instead, Class II responders exhibited a significantly stronger inhibition of cycle frequency in response to 5-HT alone as compared to when 5-HT$_{2\beta Pan}$ was blocked. This pronounced change in the 5-HT effect in Class II responders strongly suggests that 5-HT$_{2\beta Pan}$ antagonists are altering the 5-HT response. Once 5-HT$_{2\beta Pan}$ receptors were pharmacologically blocked, both classes of preparation showed a highly stereotyped, sustained inhibition in response to 5-HT (see Figure 3-3A). This inhibition likely stems from circuit effects resulting from silencing of the VD neuron in both Classes (see below).

Again, neither antagonist significantly altered the 5-HT-induced silencing of VD (Figure 3-4 C,D) or excitation of IC (n =3, data not shown) in Class II responders. These
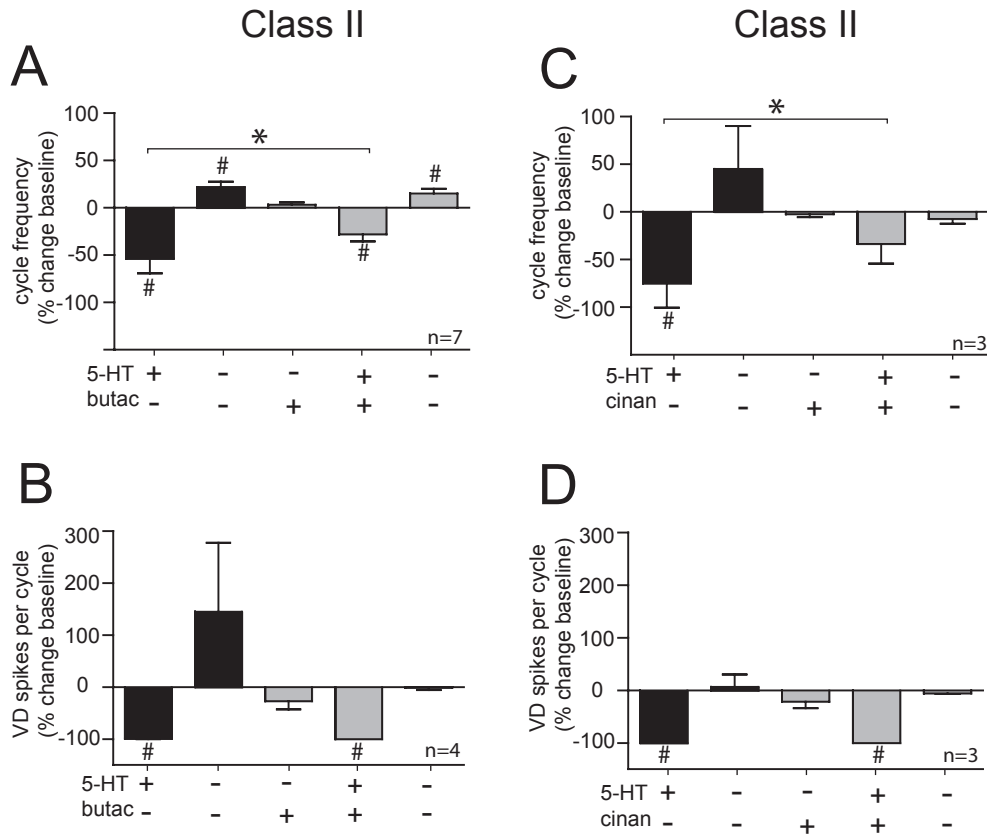
Figure 3-4: In class II preparations 5-HT application slows the pyloric rhythm via a mechanism partially involving 5-HT$_{2\beta Pan}$. The 5-HT inhibition of VD is unchanged when 5-HT$_{2\beta Pan}$ is blocked. **A**: Cycle frequency is significantly slowed by application of 10μM 5-HT. This effect is partially blocked by the 5-HT$_{2\beta Pan}$ antagonist butaclamol. **B**: Similar results were obtained with cinanserin, another 5-HT$_{2\beta Pan}$ blocker. **C, D**: The VD neuron's response to 5-HT is unchanged in these preparations; spiking is completely blocked in response to 5-HT regardless of 5-HT$_{2\beta Pan}$ antagonist presence. # p<0.05 vs. baseline; *p<0.05 for indicated comparison.

data suggest that 5-HT$_{2\beta Pan}$ receptors are not expressed in the VD or IC neuropil in Class II responders. Since the direct effect of 5-HT on the AB and the lack of effect on the PD and PY was previously only reported for Class I responders (Flamm and Harris-Warrick, 1986b; Ayali and Harris-Warrick, 1999), it is not yet clear where within the pyloric circuit 5-HT$_{2\beta Pan}$ receptors are located in Class II responders.

Interestingly, the data in Figures 3-3B,C and 3-4A,B (note difference in scales) show that when 5-HT$_{2\beta Pan}$ receptors are pharmacologically removed, there is no longer a significant difference in either the magnitude (p>0.4) or time course of the 5-HT-induced decrease in cycle frequency between Class I and Class II responders. In the presence of 5-HT$_{2\beta Pan}$ antagonists, 5-HT reduced the average cycle frequency of Class I and Class II responders relative to baseline by 22±6% (p<0.005) and 29±8% (p<0.002), respectively. This suggests that the difference between Class I and Class II responders is most likely associated with the 5-HT$_{2\beta Pan}$ transduction cascade.

5-HT$_{1\alpha Pan}$ may mediate a slow increase in cycle frequency but not silencing of VD

We next examined the role of the 5-HT$_{1\alpha Pan}$ receptor in mediating the 5-HT effect on cycle frequency. Because we were unable to identify specific antagonists for 5-HT$_{1\alpha Pan}$, we used an agonist, mCPP, to investigate the role of this receptor in mediating 5-HT modulation of the pyloric circuit. While mCPP is a relatively weak agonist of 5-HT$_{1\alpha Pan}$, it was the only one tested that had no activity on 5-HT$_{2\beta Pan}$ (Chapter 2). This criterion was important since we have shown that 5-HT$_{2\beta Pan}$ activation contributes significantly to the 5-HT effect in both Class I and Class II preparations.

Figure 3-5A,B shows that 100μM mCPP increased mean cycle frequency by 37±8%. As expected, this effect was not blocked by the 5-HT$_{2\beta Pan}$ antagonist (+)butaclamol (10μM). The mCPP-elicited increase in cycle frequency occurred for both Class I (n=2) and Class II (n=2) responders. These data suggest that 5-HT$_{1\alpha Pan}$ receptors

may play a role in mediating the 5-HT-induced change in cycle frequency. Similar to preparations exposed to 5-HT, cycle frequency after one hour of wash following mCPP was significantly faster than the initial baseline. The agonist had no significant effect on the number of VD spikes per cycle (Figure 3-5C), suggesting that the $5\text{-HT}_{1\alpha Pan}$ receptor does not mediate the 5-HT inhibition of VD. mCPP also did not mimic the 5-HT-elicited increase in IC firing (n=2, not shown).

The time course of the change in cycle frequency is different when elicited by 5-HT versus mCPP. 5-HT (10μM) effects were observed within 30s of the drug's entry to the bath in 16 of 18 preparations (Figure 3-1C) and in ~78% of our preparations, we measured the peak effect of 5-HT within 1 minute of 5-HT application. However, 100μM mCPP evoked a much slower change in cycle frequency. The peak mCPP effect always occurred after 1min of application, and in 3 of 4 preparations, a stable peak was not reached before washout of the drug (Figure 3-5A1). Furthermore, the time course of the mCPP response varied with the preparation (see Figure 3-5A1 center vs. A2). This variation may be related to the pyloric cycle frequency before mCPP application, however, further studies are required to test this correlation.

The slow rise in cycle frequency elicited by mCPP may contribute to a late increase in cycle frequency observed in response to 5-HT in some preparations. However, in most preparations this receptor would not significantly contribute to the peak 5-HT effect (compare Figures 3-1C and 3-5A). Indeed, when $5\text{-HT}_{2\beta Pan}$ is blocked with antagonists, 60% of Class II and only 20% of Class I responders show a modest cycle frequency increase in the last minute of 5-HT application. This difference may represent variability in the localization or function of $5\text{-HT}_{1\alpha Pan}$ in these preparations.

Cycle frequency responses to 5-HT in both Class I and Class II responders is therefore mediated by several coexisting mechanisms. These operate with different time courses and may involve changes in localization, function or expression of multiple
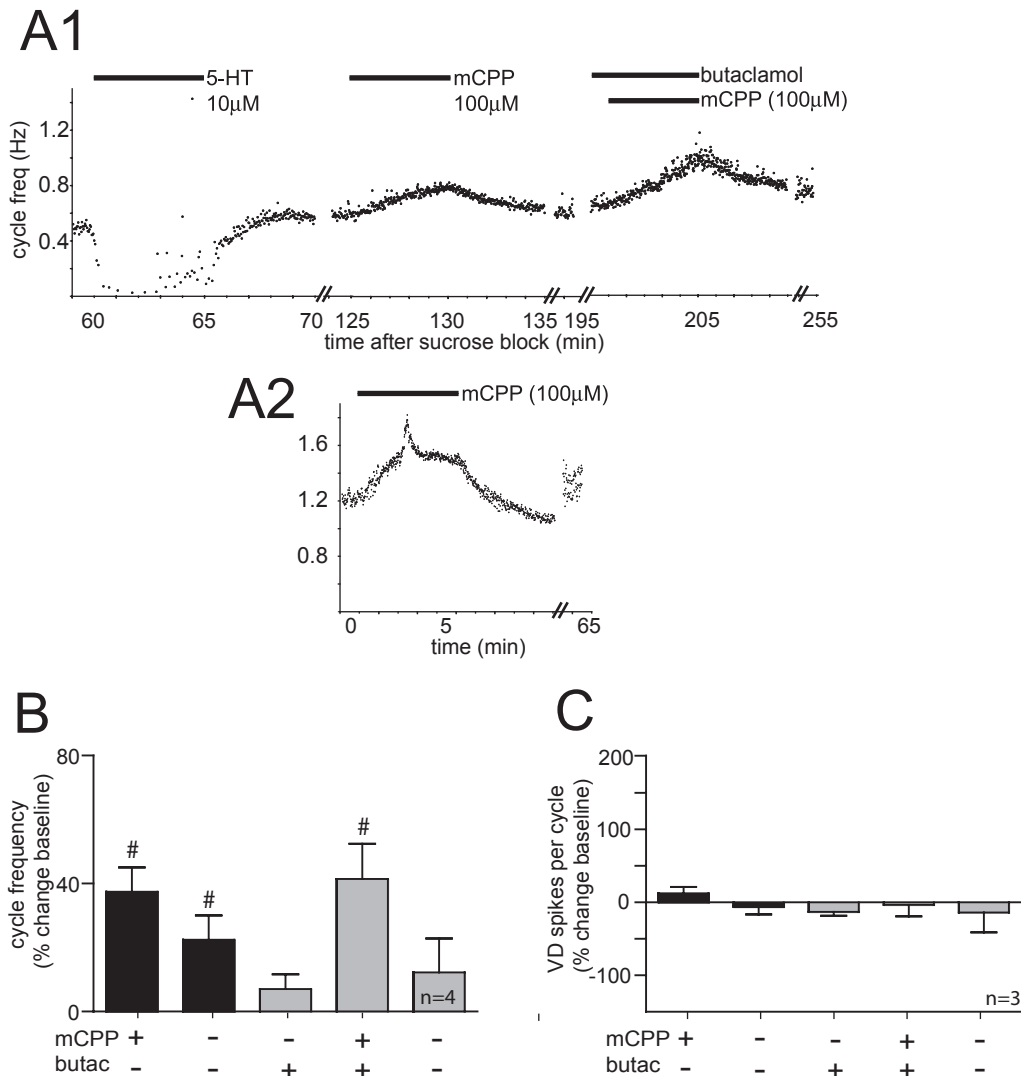
Figure 3-5: Cycle frequency may be increased via activation of 5-HT$_{1\alpha Pan}$. Serotonergic inhibition of VD does not occur via 5-HT$_{1\alpha Pan}$. **A1**: Application of 100μM mCPP, a 5-HT$_{1\alpha Pan}$ agonist, results in an increase of cycle frequency regardless of the individual preparation's response to 5-HT. In this example, 10μM 5-HT application results in a significant slowing of the rhythm (left). When mCPP is applied, however, the cycle frequency is increased (middle). This increase is not affected by the 5-HT$_{2\beta Pan}$ blocker (+)butaclamol (10μM, right). **A2**: The mCPP effect occurred with a faster rise in one of four preparations. **B**: 100μM mCPP significantly increases cycle frequency. Cycle frequency is increased over baseline after one hour of wash following mCPP application. The mCPP effect is not changed in the presence of the 5-HT$_{2\beta Pan}$ blocker, (+)butaclamol. **C**: VD spiking is not changed by mCPP application. # $p < 0.05$ vs. baseline.

5-HT receptors. 5-HT$_{2\beta Pan}$ on AB may be responsible for immediate cycle frequency increases in Class I preparations and may also contribute to the immediate 5-HT effect in Class II preparations although the localization of 5-HT$_{2\beta Pan}$ in Class II responders is undetermined. Both classes of preparations are subject to a sustained decrease in cycle frequency probably resulting from circuit effects of 5-HT silencing of the VD neuron which is unmasked when 5-HT$_{2\beta Pan}$ receptors are blocked. Finally, a later, slow increase in cycle frequency may be mediated by 5-HT$_{1\alpha Pan}$ in some preparations.

Discussion

Multiple receptors mediate 5-HT effects on the pyloric circuit

By combining putative 5-HT receptor agonists and antagonists, Zhang and Harris-Warrick (1994) demonstrated that 5-HT effects on pyloric neurons in the crab, *Cancer borealis*, are mediated by at least three distinct 5-HT receptor types. Multiple receptor types have also been proposed to be involved in differential modulation of the lateral giant escape circuit in crayfish (Yeh et al., 1997; Teshiba et al., 2001) and in crayfish behavior (Tierney and Mangiamele, 2001; Tierney et al., 2004). It was not surprising, therefore, that we found a role for both 5-HT$_{2\beta Pan}$ and 5-HT$_{1\alpha Pan}$ in modulating the pyloric circuit. In addition, we have evidence for at least one additional, as yet uncharacterized, 5-HT receptor in this system.

5-HT-mediated changes in cycle frequency

Using antagonists that block 5-HT$_{2\beta Pan}$ and not 5-HT$_{1\alpha Pan}$, we found that 5-HT$_{2\beta Pan}$ appears to play opposing roles in modulating cycle frequency in a state-dependent manner. In the presence of 5-HT$_{2\beta Pan}$ antagonists, 5-HT consistently decreased cycle frequency in a stereotyped manner. We suggest 5-HT mediates its effects on cycle frequency via unknown receptors on the VD neuron as well as 5-HT$_{2\beta Pan}$ receptors

located on the AB neuron in Class I responders and on unknown cells in Class II responders.

5-HT acts directly on the AB pacemaker neuron to increase cycle frequency in Class I preparations (Flamm and Harris-Warrick, 1986b; Ayali and Harris-Warrick, 1999). Our data suggest that $5\text{-HT}_{2\beta Pan}$ may mediate this effect. A similar result was found in the crab, *Cancer borealis*, where PD/AB bursting elicited by 5-HT was blocked by 20μM cinanserin (Zhang and Harris-Warrick, 1994), a $5\text{-HT}_{2\beta Pan}$ antagonist. The $5\text{-HT}_{2\beta Pan}$ receptor contributes to a 5-HT-mediated decrease of cycle frequency in Class II preparations, but its cellular distribution in this subset of preparations is currently unknown. It is important to note that at least three arthropod 5-HT receptors remain uncharacterized and may also be blocked by the drugs used here. If the similarity of pharmacological profiles of two *Drosophila* 5-HT_1 receptor paralogs (Saudou et al., 1992) is any indication, we would at least expect $5\text{-HT}_{2\alpha Pan}$, which is as yet uncharacterized and may be expressed in the STG, to be at least partially blocked by the antagonists used here to block $5\text{-HT}_{2\beta Pan}$. However, activity of these drugs at receptors more distantly related to $5\text{-HT}_{2\beta Pan}$ is not precluded but has been eliminated for at least one unknown 5-HT receptor expressed by the VD neuron (see below).

In the presence of $5\text{-HT}_{2\beta Pan}$ antagonists, 5-HT produces a stereotyped decrease in cycle frequency. Weaver and Hooper (2003) demonstrated that in intact preparations the activity of the VD follower neuron was important to maintaining the cycle frequency of the pacemaker ensemble in the frequency range examined here. For example, if the average cycle frequency was 1Hz, temporary removal of the VD by hyperpolarizing current injection reduced cycle frequency to 0.57Hz. In addition, the slower the intact network cycle frequency, the greater was the effect of removing the VD follower neuron. Thus, the authors suggest that VD activity governs cycle frequency to maintain an acceptable pattern of rhythmic activity via feedback mechanisms. It was also previously

shown that 5-HT acts to directly hyperpolarize the VD by 8-10mV (Flamm and Harris-Warrick, 1986b). Thus, it is possible that when 5-HT$_{2\beta Pan}$ receptors are pharmacologically removed, 5-HT reduces cycle frequency via its actions on unknown receptors on the VD. The hyperpolarization of VD by 5-HT acts indirectly to reduce cycle frequency because VD can no longer perform its normal governance role in the circuit. Removing the 5-HT$_{2\beta Pan}$ receptors therefore unmasks the VD component of cycle frequency modulation where the 5-HT effect is mediated by a receptor other than 5-HT$_{2\beta Pan}$. In our experiments, when 5-HT plus a 5-HT$_{2\beta Pan}$ antagonist were added to the bath, average cycle frequency was significantly reduced from 1.06 ± 0.05 Hz to 0.80 ± 0.07 Hz (n=17, $p<10^{-5}$). The difference in magnitude of these effects may be due to our working in sucrose-blocked preparations while Weaver and Hooper (2003) used intact preparations in their studies.

Alternatively, it is possible that our antagonists incompletely blocked 5-HT$_{2\beta Pan}$ receptors, and that these receptors are solely responsible for the 5-HT-induced change in cycle frequency. This is not likely, however, because the antagonists used are very potent with IC$_{50}$ values that are 10 and 100 times lower than the concentration applied to the preparation. Although (+)butaclamol and cinanserin block only 48 and 73% of the 5-HT effect at 5-HT$_{2\beta Pan}$ expressed in cell culture (Chapter 2), there was no apparent difference in their effects on pyloric neurons when applied at 10μM. This suggests that this concentration of either antagonist had achieved maximum block of 5-HT$_{2\beta Pan}$ receptors in the physiological preparation.

Regardless of the directionality of the 5-HT effect in a given preparation, application of mCPP always increased cycle frequency but with a different time course. 5-HT$_{1\alpha Pan}$ may therefore contribute to a later component of the 5-HT effect. In the crab STNS mCPP did not elicit 5-HT-like effects from pyloric cells but the maximum mCPP concentration tested was 50μM (Zhang and Harris-Warrick, 1994). The potency and efficacy of mCPP are highly conserved between 5-HT$_{1\alpha}$ from *Panulirus* and *Procambarus*

clarkii (Chapter 2) and if this conservation is maintained in the crab, 50μM may not have been sufficient to elicit a significant response. Indeed, in our experiments we did not observe any change in cycle frequency in response to 10μM mCPP (n=2, data not shown). Because several 5-HT receptors remain uncharacterized, we cannot rule out the action of these drugs at other sites, especially considering the high concentrations of mCPP required to elicit a response. In addition, mCPP could also be acting at other aminergic receptors to elicit its effects on cycle frequency. We have, however, ruled out mCPP activation of the uncharacterized 5-HT receptor expressed by the VD neuron.

The VD 5-HT receptor

The VD neuron is strongly inhibited by 5-HT application. This inhibition cannot be blocked with 5-HT$_{2\beta Pan}$ antagonists, nor can it be elicited with the 5-HT$_{1\alpha Pan}$ agonist. This effect is therefore mediated by neither of these receptors and must occur via a crustacean 5-HT receptor that remains uncharacterized. We believe that this is most likely the 5-HT$_7$ receptor because none of the drugs characterized at 5-HT$_{2\beta Pan}$ or 5-HT$_{1\alpha Pan}$ had any effect on the VD. The only 5-HT receptor paralogs from the same arthropod species that have been characterized are 5-HT$_{1\alpha}$ and 5-HT$_{1\beta}$ from *Drosophila* and these showed highly similar binding properties when tested with a suite of drugs. If this conservation is typical for 5-HT receptor paralogs in arthropods, the drugs used here may have had some effect at 5-HT$_{2\alpha Pan}$ and 5-HT$_{1\beta Pan}$ in addition to the characterized 5-HT$_{2\beta Pan}$ and 5-HT$_{1\alpha Pan}$. The only known arthropod 5-HT receptor remaining is 5-HT$_7$ although a completely new subtype cannot be ruled out (Clark et al., 2004). Interestingly, Clark et al. (2004) found immunocytochemical evidence of 5-HT$_{2\beta Pan}$ expression in all STG somata, including VD. Indeed, the VD may synthesize this receptor but insert functional protein only in distal membranes such as at neuromuscular junctions. In

this case, 5-HT application to the isolated STG would not activate 5-HT$_{2\beta Pan}$ receptors expressed by VD.

The pyloric circuit functions in two distinct states that are differentially modulated by 5-HT

State-dependent neuromodulatory effects have been described in the lateral giant escape circuit of crayfish where the circuit is facilitated by 5-HT in socially dominant animals and inhibited in subordinate animals. These effects can be differentially activated with agonist drugs targeted to different 5-HT receptors (Yeh et al., 1997). Additionally, the directionality of the lateral giant circuit's response to 5-HT depends on the rate and concentration of 5-HT application (Teshiba et al., 2001), supporting the notion of multiple receptors with distinct sensitivities and transduction mechanisms. In the STG of the crab, the activity history and state of the gastropyloric 2 receptor neuron determined its response to neuromodulatory input (Birmingham et al., 2003).

Previous studies have shown that superfusion of 5-HT to a sucrose-blocked STG can result in acceleration or slowing of the pyloric cycle frequency (Beltz et al., 1984; Flamm and Harris-Warrick, 1986a). Indeed, we found that STNS preparations existed in two distict states defined by cycle frequency of the intact preparation and directionality of the 5-HT response. Descending neuromodulation must be partially responsible for maintaining these states as the initial discrepancy in cycle frequency is lost when the preparation is deafferented by sucrose block. However, 5-HT elicits opposing responses even after sucrose block, indicating that intrinsic properties of the circuit also change to establish these two distinct states. State-dependent changes in response to a modulator could occur as a result of changes in expression levels of receptors with opposing effects within the same cell, such as AB, in the circuit. Indeed, identified pyloric neurons respond differently to 5-HT when they are cultured for several days indicating that the 5-

HT response mechanisms may be plastic within the cells (Turrigiano and Marder, 1993). Changes in responses to a neuromodulator occur as a result of homeostatic mechanisms that act to maintain a functional pyloric cycle in response to varied modulatory input. Comparison of the pyloric cycle in lobsters during growth revealed that while the properties of individual cells are relatively variable, the mean phase relationships of the cells within the circuit were tightly maintained regardless of cycle frequency (Bucher et al., 2005). This stability is based on activity-dependent homeostatic regulation of pyloric neurons themselves (Turrigiano et al., 1994) and of the network (Thoby-Brisson and Simmers, 1998; Golowasch et al., 1999; Spitzer, 1999; Thoby-Brisson and Simmers, 2000; Luther et al., 2003). These homeostatic mechanisms depend, at least in part, on transcription (Thoby-Brisson and Simmers, 2000). Indeed, expression of biogenic amine receptors has been shown to change after aminergic modulation (Sanders-Bush, 1990; Wohlpart and Molinoff, 1998a; Yu et al., 1998; Riad et al., 2001). In addition, activity-independent homeostatic mechanisms contribute to the maintenance of an appropriate pyloric output (MacLean et al., 2003). Such mechanisms could, therefore, contribute to the establishment and maintenance of distinct physiological states.

Mechanisms affecting the membrane properties or synaptic connectivities of the pyloric neurons themselves could contribute to configurations giving rise to distinct states. Alternatively or in addition, modulatory terminals with connections to the pyloric circuit within the neuropil could express 5-HT receptors differentially in the two states and thereby modulate the pyloric cycle differently in the presence of 5-HT. Indeed, several modulatory neurons affecting cycling frequency of STN networks have been identified (Nusbaum and Marder, 1989; Katz and Harris-Warrick, 1990; Nagy and Cardi, 1994; Norris et al., 1996; Blitz et al., 1999; Nusbaum et al., 2001; Christie et al., 2004). Cotransmission with multiple neuromodulators has been described for many of these and is an important mechanism in structuring STG outputs (Nusbaum et al.,

2001). The MCN1$_{STG}$, a descending modulatory neuron, has been shown to exert local modulation of the crab pyloric circuit within the neuropil arbor in STG independent of the neuron's central activity (Coleman and Nusbaum, 1994). If modulatory neurons such as MCN1$_{STG}$ express axonal 5-HT receptors they could therefore modulate the pyloric cycle in response to 5-HT applied to the STG in sucrose block. Second-order aminergic modulation of descending interneurons has been described in the swim central pattern generator of the leech (Mesce et al., 2001; Crisp and Mesce, 2003). In addition, metamodulatory mechanisms have been demonstrated in several other invertebrate systems (Katz and Edwards, 1999; Mesce et al., 2001; Edwards et al., 2002). Expression of 5-HT receptors on modulatory neurons could vary in a state-dependent fashion, contributing to state-dependent 5-HT effects. Descending modulatory input could thus shape the 5-HT response by dictating 5-HT receptor expression or function at direct and metamodulatory 5-HT target sites in the circuit, giving each more or less weight in contributing to the final cycle frequency observed in 5-HT.

Our results indicate that 5-HT$_{2\beta Pan}$ activation has opposite effects on cycle frequency in the two states while 5-HT$_{1\alpha Pan}$ has the same effect but to a variable degree in different preparations. Instead of diverse targets expressing multiple receptors, the two classes of preparations and their differential 5-HT modulation could arise from receptor differences within individual cells such as the AB pacemaker. In vertebrates, 5-HT receptor expression is state-dependent (Cirelli and Tononi, 2004; Dwivedi et al., 2005) and can change as a result of receptor activation (Wohlpart and Molinoff, 1998a; Anji et al., 2001; Riad et al., 2001). This mechanism may be active at the 5-HT$_{1\alpha Pan}$ receptor in the STNS since we saw significant variability in the slow activation thought to be mediated by these receptors among preparations.

Alternatively, the same receptor subtypes could be differentially coupled to different signaling cascades in a state-dependent manner. The 5-HT$_{2\beta Pan}$ receptor could

change its contribution to the 5-HT effect in Class I versus Class II preparations by such a mechanism. The variability in G protein coupled receptor (GPCR) signaling is staggeringly complex. Besides the traditional Gα pathways, extensive signaling occurs via the G$\beta\gamma$ subunit upon receptor activation; this pathway can halt or even antagonize the Gα mediated effect (Liu et al., 1999) or activate diverse cascades that can result in altered gene expression. Second-order feedback effects of G protein mediated signaling can switch the G protein coupling of an individual receptor in mid-signal (Daaka et al., 1997; Lefkowitz et al., 2002). In addition, GPCR signals can be mediated by a myriad of G protein independent pathways such as β-arrestin, various kinases and direct receptor-receptor interactions (Heuss and Gerber, 2000; Brzostowski and Kimmel, 2001; Hansen and Schmidt, 2004; Lee and Liu, 2004). Activation of the same receptor, such as 5-HT$_{2\beta Pan}$, expressed in the same cell, AB, could therefore have very different effects depending on how the signaling machinery is configured.

Finally, uncharacterized 5-HT receptors on pyloric or modulatory neurons could contribute to the 5-HT effect. These receptors may also be targets for the drugs used in this study. Future studies to characterize additional receptors as well as strategic combinations of identified drugs should allow further dissection of 5-HT modulation of the pyloric circuit.

Conclusions

Pharmacological tools developed with cloned *Panulirus* receptors in heterologous expression systems have allowed us to investigate the role of these receptors in mediating serotonergic modulation in an intact physiological system. We have determined that 5-HT$_{2\beta Pan}$ receptors mediate an early portion of the 5-HT response. On the other hand, 5-HT$_{1\alpha Pan}$ receptors may be involved in the later portion of the 5-HT. In addition, neither of these receptors is involved in inhibition of the VD neuron by 5-HT. The pyloric circuit

exists in two distinct states in which 5-HT modulation of cycle frequency has opposing effects. Interestingly, when the 5-HT$_{2\beta Pan}$ receptors are pharmacologically blocked there is no longer a significant difference between Class I and Class II responders in the presence of 5-HT, suggesting that state determination may be associated primarily with the 5-HT$_{2\beta Pan}$ transduction cascade. Our data suggest that 5-HT$_{2\beta Pan}$ receptors are located on AB in Class I preparations. In addition, both 5-HT$_{2\beta Pan}$ and 5-HT$_{1\alpha Pan}$ may be expressed by multiple neurons that contribute to regulation of pyloric cycle frequency such as the AB pacemaker itself and local neurites of modulatory neurons. We propose that the two states arise as a result of descending neuromodulatory input which reconfigures 5-HT receptors, perhaps by changes in expression at the multiple sites affecting cycle frequency or by reconfiguring receptor signaling within the same target cells. These hypotheses can be addressed in future studies by examining 5-HT receptor expression patterns and signaling in individual identified neurons of the STNS and by combining pharmacology with existing physiological techniques.

Chapter 4   Localization and expression of 5-HT$_{1\alpha crust}$ in the crayfish nerve cord

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Introduction

The biogenic amine, serotonin (5-HT), is an important modulator of sensory and motor function across phyla. The serotonergic system of mammals has been linked to regulation of many basic biological functions and it is known to play a role in several human disorders, including major depression and suicidal behavior (reviewed in Mann, 1999). In addition, serotonin levels and serotonin receptor activity are sensitive to stress, including social stress (reviewed in Chaouloff et al., 1999). The serotonergic system in crustaceans has been well described (Beltz and Kravitz, 1983; Real and Czternasty, 1990; Harzsch and Waloszek, 2000; Antonsen and Paul, 2001) and many individual cells have been characterized with respect to their physiology and their response to serotonin (reviewed in Kravitz, 2000). In crustaceans, serotonin modulates sensori-motor systems for locomotion, posture, escape, digestion, stress, and aggression (Livingstone et al., 1980; Kravitz, 1988; Rossi-Durand, 1993; Edwards et al., 1999). Olfaction, proprioception, mechanosensation, heart rate and vision are also modulated by serotonin, as is neurogenesis in juvenile crustacean brains (Sandeman et al., 1988; Wilkens, 1999; Benton and Beltz, 2001; Escamilla-Chimal et al., 2001). In crayfish, serotonergic modulatory effects have been found to depend on the pattern of serotonin exposure

(Teshiba et al., 2001) and the social status and experience of the crayfish (Yeh et al., 1996; Yeh et al., 1997). The differences in the effects of serotonin with exposure pattern or social status appear to reflect the actions of different serotonin receptors. Although pharmacological studies indicate that three or more 5-HT receptors are present in the crustacean CNS (Zhang and Harris-Warrick, 1994; Yeh et al., 1996), only two, $5\text{-HT}_{1\alpha\text{crust}}$, and $5\text{-HT}_{2\beta\text{crust}}$ (Clark et al., 2004; Sosa et al., 2004, Chapter 2) have been described.

We (Sosa et al., 2004) cloned the first putative crustacean 5-HT type 1 receptor ($5\text{-HT}_{1\alpha\text{crust}}$) from crayfish ($5\text{-HT}_{1\alpha\text{Pro}}$), prawn ($5\text{-HT}_{1\alpha\text{Mac}}$) and spiny lobster ($5\text{-HT}_{1\alpha\text{Pan}}$) and generated polyclonal antibodies against conserved sequences in all three species. When expressed in HEK cells, the cloned receptor mediates inhibition of cAMP production presumably via activation of $G\alpha_{i/o}$ like all other type 1 5-HT receptors (Chapter 2). A preliminary immunocytochemical localization of $5\text{-HT}_{1\alpha\text{crust}}$ in crayfish and prawn thoracic ganglia (Sosa et al., 2004) showed that these two species show similar patterns of $5\text{-HT}_{1\alpha\text{crust}}$ expression in the thoracic nerve cord.

Here we greatly expand the immunocytochemistry study to show a detailed map of $5\text{-HT}_{1\alpha\text{crust}}$ immunoreactivity ($5\text{-HT}_{1\alpha\text{crust}}$ir) throughout the entire crayfish nerve cord and on abdominal superficial flexor muscles and blood vessels. $5\text{-HT}_{1\alpha\text{crust}}$ is widely expressed in both somata and neuropil throughout the crayfish nervous system. This map will aid in a molecular analysis of the role of serotonergic modulation in identified circuits and cells. We found that a wide range of variation occurs between animals both in immunoreactivity and in the level of receptor mRNA expression. Curiously, these two parameters are not correlated in an individual. The variability in receptor mRNA and immunoreactivity also did not correlate directly with gender, diet, molt status, circadian rhythm or individual social experience.

Methods and Materials

Immunocytochemistry

Crayfish (*Procambarus clarkii*) measuring 3-5cm rostrum to telson were obtained from Atchafalaya Biological Supply (Raceland, LA). They were kept communally in 40-L tanks with continual filtration and aeration in a 12/12 light cycle. Many individual shelters made of PVC tubing were provided. Animals were fed shrimp pellets (AQUADINE Nutritional System, Heraldsburg, CA) and/or water plants twice a week.

Generation of the $5\text{-HT}_{1\alpha\text{crust}}$ antibody is described in detail in Sosa et al. (2004). Briefly, $5\text{-HT}_{1\alpha\text{crust}}$ cDNA was sequenced completely from lobster; for crayfish and prawn, the sequence spanning the region between transmembrane domains III–VII was obtained. The sequence is highly stereotypical of G-protein coupled receptors and was determined to represent a 5-HT_1 type receptor based on sequence identity in key domains with other characterized invertebrate receptors. $5\text{-HT}_{1\alpha\text{crust}}$ amino acid sequences from crayfish, lobster and prawn were aligned and two peptides representing conserved, highly charged non-transmembrane regions specific to the receptors in these three species were identified. Antibodies against these peptides were generated commercially (Bethyl Laboratories, Montgomery, TX) and used as follows.

Animals were chilled in ice water for 15min prior to dissection. The time of dissection, size, gender, molt status and gut contents of each animal was noted. They were pinned out in crayfish saline (in mM: 202 NaCl; 5.37 KCl; 13.53 $CaCl_2$; 2.6 $MgCl_2$; 2.4 HEPES; pH 7.4) and the nerve cord was exposed by dorsal dissection. The sheath was removed from the dorsal side of each ganglion. The nerve cord was immediately placed in fresh 4% paraformaldehyde fixative, pH 7.4 in crayfish saline, and left overnight at 4°C. Nerve cords were rinsed in distilled water and washed 6x 1 hour in PBTX (0.1M sodium phosphate, pH 7.4 (PB) + 0.25% TritonX-100). Preparations were

then washed twice in water and dehydrated through an ethanol series (8min each: 30, 50,
70, 80, 90, 95, 100%) then rehydrated (100, 70, 50, 30% ethanol, PB). Nerve cords were
incubated two nights at 4°C with shaking in PBTX with 2μg/ml rabbit anti-5-HT$_{1\alpha crust}$
(Sosa et al., 2004) and, for double labeling with anti-serotonin, a 1:50 dilution of a mouse
anti-serotonin monoclonal antibody (DAKO Corp, Glostrup, Denmark). Nerve cords
were washed 6x 1hr at 4°C in PBTX then incubated in PBTX with a 1:50 dilution each
of Texas Red goat anti-rabbit and Oregon Green goat anti-mouse (Molecular Probes,
Eugene, OR). After incubating overnight at 4°C the preparations were washed 6x one
hour in PBTX, dehydrated sequentially up to 100% ethanol and mounted in methyl
salicylate (Sigma, St. Louis, MO).

All 5-HT$_{1\alpha crust}$ immunoreactivity was lost when the primary antibody was
preabsorbed with peptide (1:20 w/w ratio) for 7hr at room temperature prior to
application to tissue and when primary antibody was omitted from the procedure (data
not shown). The immunocytochemistry data presented here are based on observation of
over 100 preparations.

For double-label with anti-5-HT$_{1\alpha crust}$ and propidium iodide, 5μM propidium
iodide (Sigma, St. Louis, MO) was included in the primary antibody incubation mixture.
Preparations were treated as above (with only Oregon Green goat anti-rabbit secondary)
and mounted in methyl salicylate.

For immunocytochemistry of superficial flexor muscles with anti-5-HT$_{1\alpha crust}$, the
muscles and nervous system were exposed by dorsal dissection. Segments of superficial
flexor muscles attached to abdominal cuticle were pinned to small wafers of sylgard
(Dow Corning, Midland, MI) using glass pins. Square segments were cut out of the
cuticle above the sylgard wafer and the whole combination was placed directly into fresh
fixative. Preparations were treated for 5-HT$_{1\alpha crust}$ immunoreactivity as above. After the
final incubation, preparations were washed 4x 1hr in PBTX, 2x 1hr in PB, removed from

the sylgard wafers and mounted directly in glycerol mounting medium (9ml glycerol + 1ml crayfish saline, pH adjusted to 8-9 with sodium carbonate-bicarbonate buffer, pH 9.2).

All preparations were stored at -20°C until they were imaged on a Zeiss LSM 510 confocal microscope. Most images were obtained using a Zeiss Fluar 20x/0.75 UV objective with digital zoom ranging from 0.7 to 3x. Fine detail was imaged using a 63x C-Apochromat 1.2W objective with digital zoom up to 3x or a Plan-Neofluar 40x/1.3Oil objective with 2x digital zoom. The subesophageal ganglion collage was imaged with a Zeiss 10x CP Achromat 0.25 Ph1 with a 0.7x digital zoom. Confocal stacks were reconstructed and analyzed using Adobe Photoshop 7.0 on a Macintosh G4 Powerbook. Colors were digitally adjusted to represent 5-HT$_{1\alpha crust}$ir as green in all dual-color images for consistency and clarity.

Quantitative RT-PCR

Crayfish were kept isolated in 1L tanks for 4 weeks. Animals were sacrificed by cutting off the head (without chill). The brain and circumesophageal ganglion were immediately removed and placed in a tared microcentrifuge tube on dry ice. Thoracic ganglia 4 and 5 from the same animal were desheathed on the dorsal side and placed in fresh paraformaldehyde for immunocytochemistry processing as above. For quantitative RT-PCR total RNA was isolated from each brain using 800μl Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Phase separation steps were aided using PhaseLock Gel (Eppendorf AG, Hamburg, Germany) and RNA was precipitated with GlycoBlue Coprecipitant (Ambion, Austin, TX). The RNA concentration was determined by OD measurement. Remaining genomic DNA was degraded using a MessageClean Kit (GenHunter, Nashville, TN) and samples were stored at –80°C. Complementary first-strand DNA was synthesized as follows: 5μl (of

20μl total, ~5μg) cleaned mRNA was combined with 6.5μl sterile distilled water, 1.0μl dNTPs (10mM each ATP, GTP, CTP, TTP, Roche, Mannheim, Germany) and 0.5μl random hexanucleotide mix (Roche, Mannheim, Germany), this mixture was incubated at 65°C for 10min, chilled on ice, centrifuged and allowed to warm to room temperature. Superscript II reverse transcriptase was then added (1μl) with 2μl dithiothreotol and 4μl 5x buffer both provided with the enzyme (Gibco/Invitrogen, Carlsbad, CA) the reaction was allowed to proceed for 1.5hr at 37°C, then the enzyme was inactivated by heating to 70°C for 10min, the samples were stored at –20°C until real-time PCR analysis. Samples were analyzed on an ABI Prism 7700 Sequence Detector using default parameters in triplicate for 5-HT$_{1\alpha Pro}$ mRNA and, separately, rRNA as an internal control. The probe used for 5-HT$_{1\alpha Pro}$ detection was 6FAM-AAGAAAGAGGCCAAGC AGAAAAAAAATAGCAACAA-TAMRA (custom synthesized by Applied Biosystems, Foster City, CA) with the following primers 5'GGGTCGCCGGACAAGTC3' and 5'CTCGGAAATGTGGGACGAAA3' (custom synthesized by Sigma Genosys, The Woodlands, TX). This probe/primer combination was designed based on the crayfish-specific sequence of the 5-HT$_{1\alpha crust}$ receptor (5-HT$_{1\alpha Pro}$) using PrimerExpress software (Applied Biosystems, Foster City, CA). The internal control rRNA was detected using TaqMan rRNA Control Reagents with a VIC probe using probe and primer concentrations suggested by the manufacturer (Applied Biosystems, Foster City, CA). Each 30μl PCR reaction quantifying 5-HT$_{1\alpha Pro}$ contained 15μl TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA), 1.2μl of each primer (20μM), 0.3μl probe (10μM), 2μl cDNA sample (1/10 dilution) and 10.3μl sterile distilled water. The number of PCR cycles required to cross a threshold (C_t) of fluorescence were determined for 5-HT$_{1\alpha Pro}$ mRNA and control rRNA. Relative amounts of 5-HT$_{1\alpha Pro}$ mRNA and rRNA were calculated by comparison of C_t to a standard curve of crayfish cDNA run on the same

plate and each 5-HT$_{1\alpha Pro}$ concentration was normalized to the rRNA concentration in the same sample.

For 5-HT$_{1\alpha Pro}$ mRNA quantification in dominant and subordinate crayfish, animals were kept in pairs in 15-L aquaria, one animal in each pair was marked with a spot of liquid paper on the carapace. Animals were scored every morning for social status. An animal was said to be dominant if it was in, on or next to the single shelter at the center of the tank or if it showed aggressive behavior (approach, attack) towards its partner. Animals were considered subordinate if they were at the edges of the tank or retreating from the other animal. Time lapse filming of pairs over >24 hours has shown that these are good indicators of social status (unpublished observations). After 14 days of unchanged status both animals were sacrificed by cutting off the head (without chill), the nerve cord (brain – A6) was quickly removed an placed in a tared microcentrifuge tube on dry ice and relative levels of 5-HT$_{1\alpha Pro}$ mRNA were quantified as above.

Results

The putative serotonin receptor 5-HT$_{1\alpha crust}$ is expressed in reproducible patterns throughout the crayfish nervous system (Figure 4-1). 5-HT$_{1\alpha crust}$ immunoreactivity (5-HT$_{1\alpha crust}$ir) appears in the cytoplasm and around the periphery of numerous somata throughout the nervous system. These somata are distributed throughout all ganglia in a typical pattern with a cluster of 5-HT$_{1\alpha crust}$ir somata on the ventral midline extending to the lateral anterior and posterior corners of the ganglion. Here, the cell clusters expand through the ganglion to include 5-HT$_{1\alpha crust}$ir somata on the dorsal surface. Motor neuron somata identified by back-fills of thoracic and abdominal peripheral nerves fail to display 5-HT$_{1\alpha crust}$ir (data not shown), suggesting that immunoreactive somata throughout the nerve cord are interneurons. In addition, somata of presumed sensory or neurosecretory neurons in nerves 1 and 2 of thoracic and abdominal ganglia express 5-HT$_{1\alpha crust}$. Nerves
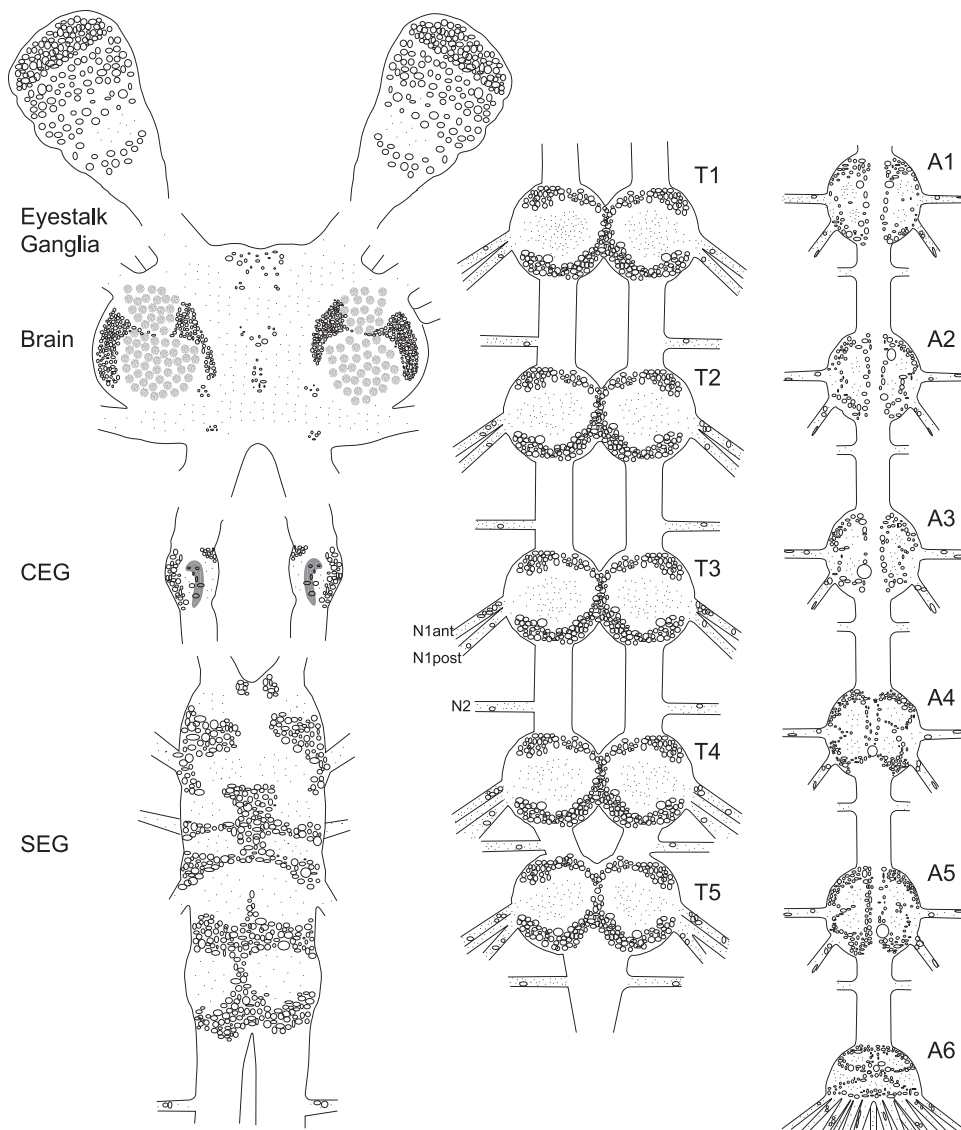
Figure 4-1: Schematic representation of 5-HT$_{1\alpha crust}$ir in the nerve cord of the crayfish, *Procambarus clarkii*. Cell bodies are represented by ovals but the number and size of these is meant to indicate the typical location of label and approximate relative numbers rather than representing actual cells in an individual preparation. Many of the cells shown, especially in the nerves, appear only in some preparations and only in some nerves. Stippling indicates staining of neuropil and neural plexus on nerves and in olfactory and accessory lobe glomeruli in the brain. The shaded area in CEG (circumesophageal ganglion) represents an unidentified structure that exhibits diffuse 5-HT$_{1\alpha crust}$ir. SEG, subesophageal ganglion; T1-5, thoracic ganglia; N1ant, large anterior branch of thoracic nerve 1; N1post, large posterior branch of thoracic nerve 1; N2, thoracic nerve 2; A1-6, abdominal ganglia. (Adapted from Sandeman et al., 1988 and Real & Czernasty, 1990)

1 and 2 of thoracic nerves also show 5-HT$_{1\alpha crust}$ir in a plexus in association with a serotonergic neurohormonal plexus. All nerves of abdominal ganglia show very punctate 5-HT$_{1\alpha crust}$ staining. Neuropil throughout the nerve cord labels for 5-HT$_{1\alpha crust}$ir in a punctate fashion, often in apposition or superposition with 5-HTir. Neuropil and plexus 5-HT$_{1\alpha crust}$ label is very punctate and delineates only very short stretches of neurites. 5-HT$_{1\alpha crust}$ir is uniformly dispersed through all neuropils of ganglia and differences in label could not be distinguished between different identifiable neuropil regions. These patterns of labeling are highly conserved between ganglia and between animals and will be referred to as the 'typical pattern' of labeling.

Brain

The most prominent 5-HT$_{1\alpha crust}$ir labeling in the brain (or supraesophageal ganglion) (Figure 4-2) is on the somata of cells in clusters 10 and 9&11 of the central olfactory pathway. These clusters are attached to the olfactory (OL) and accessory lobe (AL) neuropils. Many but not all cells throughout these clusters are 5-HT$_{1\alpha crust}$ir as shown by double-labeling with the nuclear marker propidium iodide (Figure 4-3A). In a typical 5-HT$_{1\alpha crust}$ir cell, labeling is irregularly distributed throughout the cytoplasm and around the periphery of the soma (Figure 4-3B-D). Propidium iodide labeling does not show flat nuclei that might represent glial cells within the clusters, indicating that the individual bright puncta around the periphery of somata are not associated with smaller cells. The 5-HT$_{1\alpha crust}$ir cells are small, approximately 5-20µm in diameter, and irregularly shaped. 5-HT$_{1\alpha crust}$ir is located in puncta throughout the cytoplasm on what may be structures of the endomembrane system (Figure 4-3E-I). Puncta also appear along the edges of the cell but without electron microscopy it cannot be determined if these represent receptors on the surface of the cell. It is also not possible to determine if all the label is associated with the cell body or with small neurites passing close to the somata. The primary neurite

Figure 4-2: 5-HT$_{1\alpha crust}$ immunoreactivity in the brain and eyestalk ganglia. **A:** 5-HT$_{1\alpha crust}$ is strongly expressed in somata in cell clusters throughout the brain and eyestalk ganglia. The somata of local olfactory interneurons in clusters 9&11 and projection olfactory interneurons in cluster 10 label strongly for 5-HT$_{1\alpha crust}$. Individual glomeruli of the olfactory (OL) and accessory (AL) lobes show punctate 5-HT$_{1\alpha crust}$ir. Other neuropil areas throughout the brain also show diffuse label as do some fibers at the base of the eyestalks. Much of the protocerebral tracts connecting the brain and eyestalk ganglia are not shown; the removed area did not show additional labeled structures beyond what can be seen at either end of the connectives. **B:** The same image showing 5-HT$_{1\alpha crust}$ immunoreactivity (green) and 5-HT immunoreactivity (red). The arrowhead indicates a small group of 5-HTir neurons including the dorsal giant neuron which exclusively provide serotonergic innervation of OL and AL. 5-HT$_{1\alpha crust}$ir somata are found in every layer of the eyestalk ganglia, these are interspersed with 5-HTir cells but the two cell types do not appear double-labeled or spatially associated. LG, lamina; ME, external medulla; MI, internal medulla; MT, medulla terminalis. This image is a digital collage of 25 confocal stacks each containing many optical slices that span the entire dorsal-ventral axis of the ganglia. The fluorescence has been digitally brightened and several pieces of surface debris have been digitally removed. Scale bar = 500 μm.
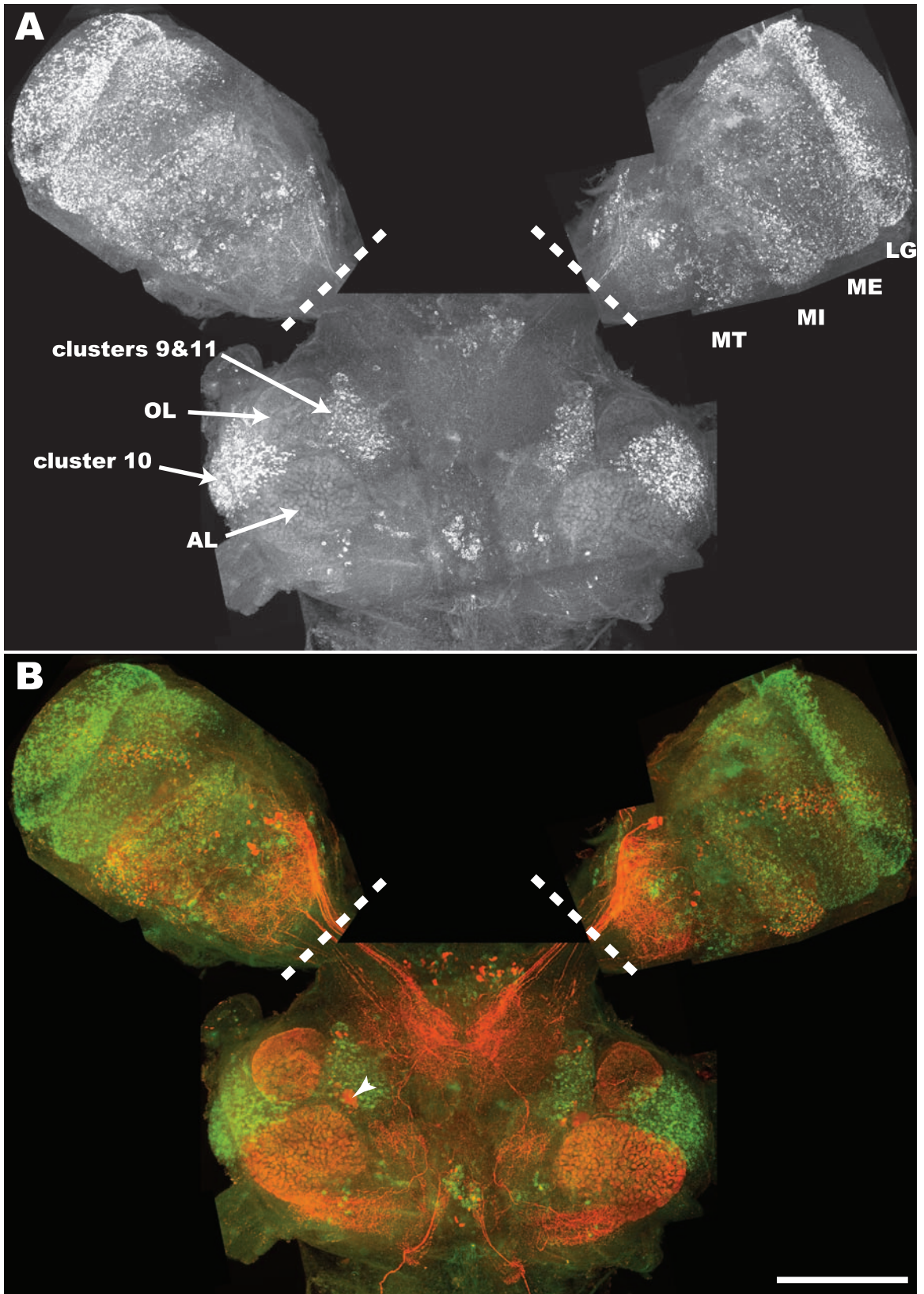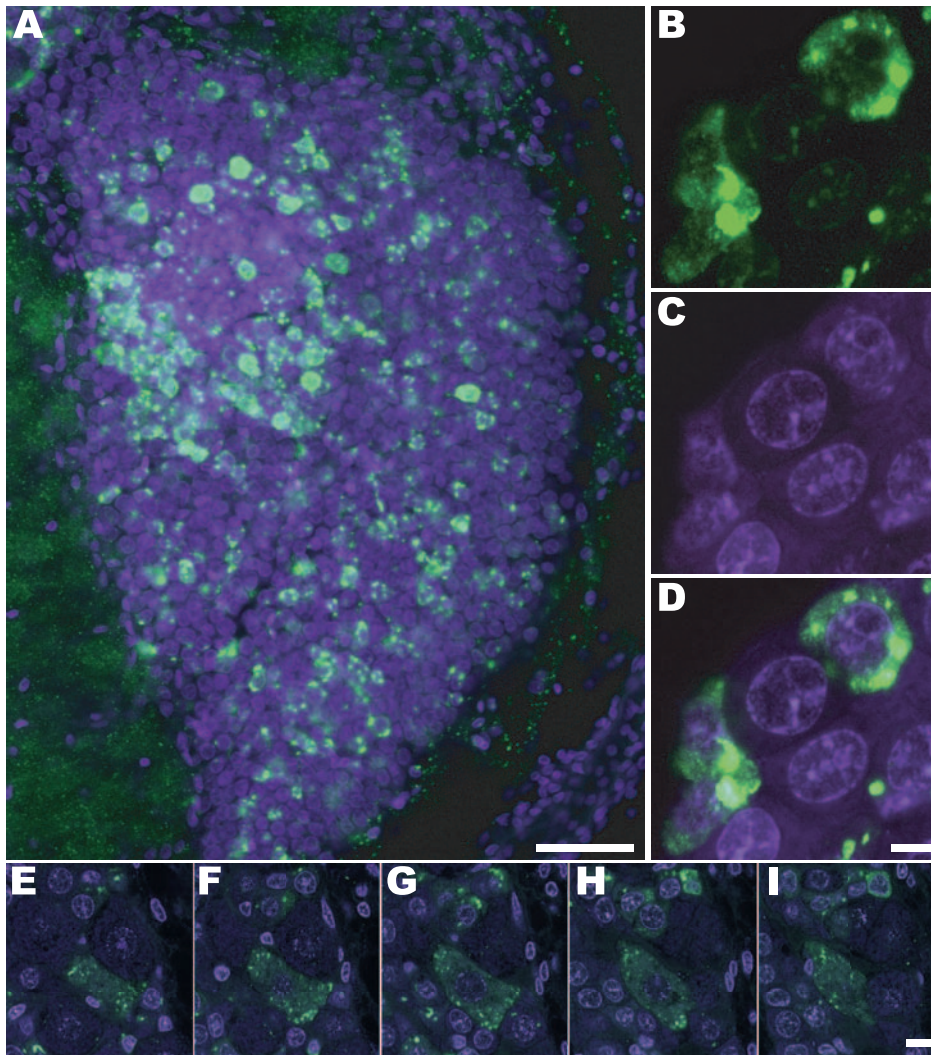
Figure 4-3: 5-HT$_{1\alpha crust}$ (green) is located in cytoplasm and on the cell membrane of olfactory interneurons in cluster 10. The nuclear marker propidium iodide (purple) was used to label all cell nuclei. **A:** A subset of cells dispersed throughout cluster 10 is 5-HT$_{1\alpha crust}$ir. Determination of the proportion of cluster cells appearing 5-HT$_{1\alpha crust}$ir is not feasible due to the high variation in labeling observed between animals (see later sections). This image is a stack of two adjacent confocal slices spanning a thickness of ~5μm. **B:** Individual 5-HT$_{1\alpha crust}$ir cells in a lateral cluster of T5, **C:** their nuclei and those of non-5-HT$_{1\alpha crust}$ir cells, **D:** merged to show the association of 5-HT$_{1\alpha crust}$ with individual nuclei. Panels B-D are digitally color-separated views of a stack of six confocal slices spanning a thickness of ~6μm. **E-I:** A series of optical slices <0.5μm thick and ~3μm apart show localization of 5-HT$_{1\alpha crust}$ puncta to the cytoplasm and around the periphery of an individual cell in a lateral cell cluster of T5. This represents the typical 5-HT$_{1\alpha crust}$ staining pattern observed in somata throughout the nerve cord. The fluorescence in all panels has been digitally brightened. Scale bars = 50μm in A; 5 m in B-D; 10μm in E-I.

of some of the 5-HT$_{1\alpha crust}$ir cells labels proximally (<10μm) to the soma but labeling does not follow the path taken by the neurites through the cluster. Occasionally, serotonergic fibers surround the cells of cluster 10. These 5-HTir fibers (Figure 4-4A, red) can be found associated with some cells that express 5-HT$_{1\alpha crust}$ (Figure 4-4A arrow) and other cells that do not (Figure 4-4A arrowhead). 5-HT$_{1\alpha crust}$ir is also superposed with 5-HTir in the neuropil glomeruli of the olfactory and accessory lobes (Figure 4-4B). In these dense neuropil structures some processes label for 5-HT only (red) or 5-HT$_{1\alpha crust}$ only (green) and many show possible colocalization of transmitter and receptor (yellow). 5-HT$_{1\alpha crust}$ staining of neuropil fibers is primarily punctate and delineates individual processes only occasionally and for very short distances. The 5-HT innervation of the OL and AL is provided exclusively by a small group of cells including the dorsal giant interneuron (DGN) (Figure 4-2, arrowhead) (Sandeman et al., 1988). 5-HT$_{1\alpha crust}$ir is not observed on the soma, primary neurite or cytoplasm of the DGN suggesting that 5-HT$_{1\alpha crust}$ label in the OL and AL neuropils is located on cells receiving synaptic or paracrine 5-HT signals from DGN. In addition to clusters 10 and 9&11, subpopulations of somata throughout all described cell clusters in the brain (Sandeman et al., 1992) also show 5-HT$_{1\alpha crust}$ir (Figure 4-2).

Eyestalk Ganglia

The eyestalks of the crayfish contain the X-organ-sinus gland complex, a prominent neurohemal organ, and the eyestalk ganglia. 5-HT$_{1\alpha crust}$ir is seen in somata of cells distributed throughout all soma clusters of the four major neuropils of the optic lobe (Figure 4-2). These cells show a typical pattern of labeling similar to cells in clusters 10 and 9&11 of the central brain (Figure 4-3B); they are small, irregularly labeled and show punctate 5-HT$_{1\alpha crust}$ir throughout the cytoplasm and along the periphery. Some 5-HT$_{1\alpha crust}$ir cells are located around a dense plexus of serotonergic fibers in the terminal
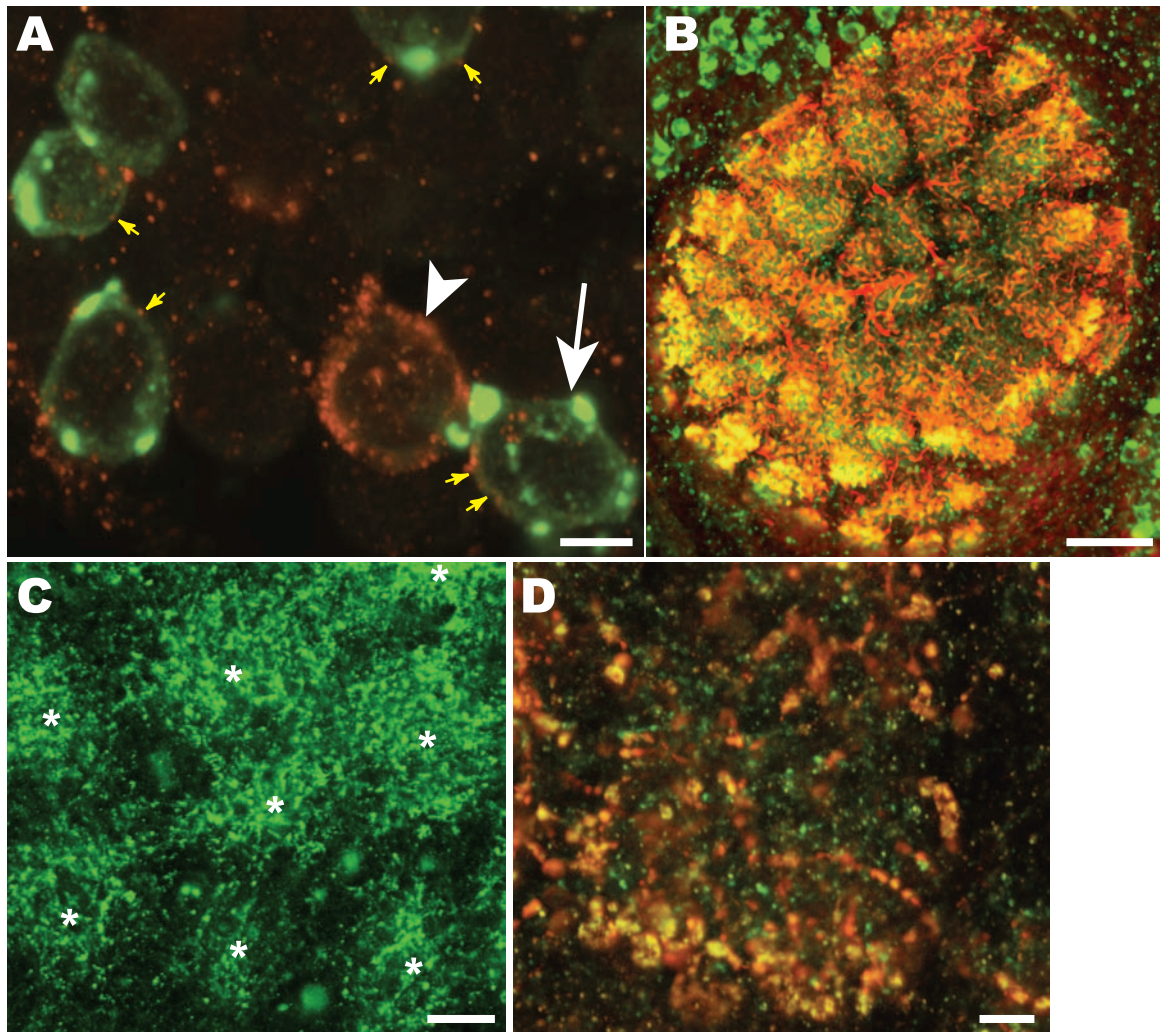
Figure 4-4: Relationship of 5-HT$_{1\alpha crust}$ir (green) and 5-HTir (red) in cluster 10 and in glomeruli of olfactory and accessory lobes. **A:** Individual somata in cluster 10 are surrounded by 5-HTir processes (red). Some of these also show 5-HT$_{1\alpha crust}$ir (white arrow) and colocalization of transmitter and receptor around the periphery of the cell (yellow arrows) while others do not express 5-HT$_{1\alpha crust}$ (arrowhead). This is a stack of 5 adjacent confocal slices spanning a thickness of ~5μm. **B:** Individual glomeruli show 5-HT$_{1\alpha crust}$ir (green) and 5-HTir (red) localized alone and superposed together (yellow). This is a stack of 4 confocal slices spanning a thickness of 10μm through the olfactory lobe. **C:** A group of glomeruli in the accessory lobe labeled only for 5-HT$_{1\alpha crust}$ir, asterisks indicate individual glomeruli. **D:** Double label of an olfactory lobe glomerulus shows processes that are only 5-HT$_{1\alpha crust}$ir (green), processes that are only 5-HTir (red) and processes where the two are superposed (yellow). 5-HT$_{1\alpha crust}$ label is located in puncta along processes. C and D are single confocal optical slices of ~1μm thickness. The fluorescence in all images has been digitally brightened. Scale bars = 5μm in A & D; 50μm in B 10μm in C.

medulla. These 5-HT$_{1\alpha crust}$ir cells are sharply separated from the 5-HTir fibers, however, indicating that they may respond to neurohormonal or paracrine 5-HT signals. 5-HT$_{1\alpha crust}$ir neurons are located throughout the internal and external medullae and in the lamina and they are interspersed with 5-HTir neurons but these two types of somata do not appear to have a specific association.

Circumesophageal and Subesophageal Ganglia

The circumesophageal ganglion (CEG) shows typical 5-HT$_{1\alpha crust}$ir in small somata located on the ventral surface and up the lateral edge towards the dorsal side of the ganglion (Figure 4-5A, B). The CEG also has a central 5-HT$_{1\alpha crust}$ir structure where the labeling appears as a smooth patch (Figure 4-5A arrow). Examination of this structure at higher magnification shows only diffuse 5-HT$_{1\alpha crust}$ label; identification of this structure will therefore require closer analysis using cell fills or electron microscopy. While the CEG contains extensive 5-HT innervation, 5-HT fibers are not associated with this central 5-HT$_{1\alpha crust}$ir structure. Punctate neuropil such as that seen throughout the nerve cord (below) is also present in the core of the ganglion.

In the subesophageal ganglion 5-HT$_{1\alpha crust}$ir is found in a typical pattern (Sosa et al., 2004) for every segment of this fused ganglion (Altman and Kien, 1987). This pattern shows 5-HT$_{1\alpha crust}$ir somata clustered along the midline axis on the ventral surface and out towards the lateral corners of each segment (Figure 4-5C, D), where they extend to the dorsal surface of the ganglion. 5-HT$_{1\alpha crust}$ir is found in puncta throughout the neuropils of the SEG and on nerves leaving the ganglion. 5-HT$_{1\alpha crust}$ir labeling patterns on the nerves of the SEG is similar to that seen on the nerves of the thoracic ganglia (see below). While 5-HTir fibers spread throughout the SEG, the 5-HT$_{1\alpha crust}$ir somata are not specifically associated with them, indicating that any functional receptors on these somata
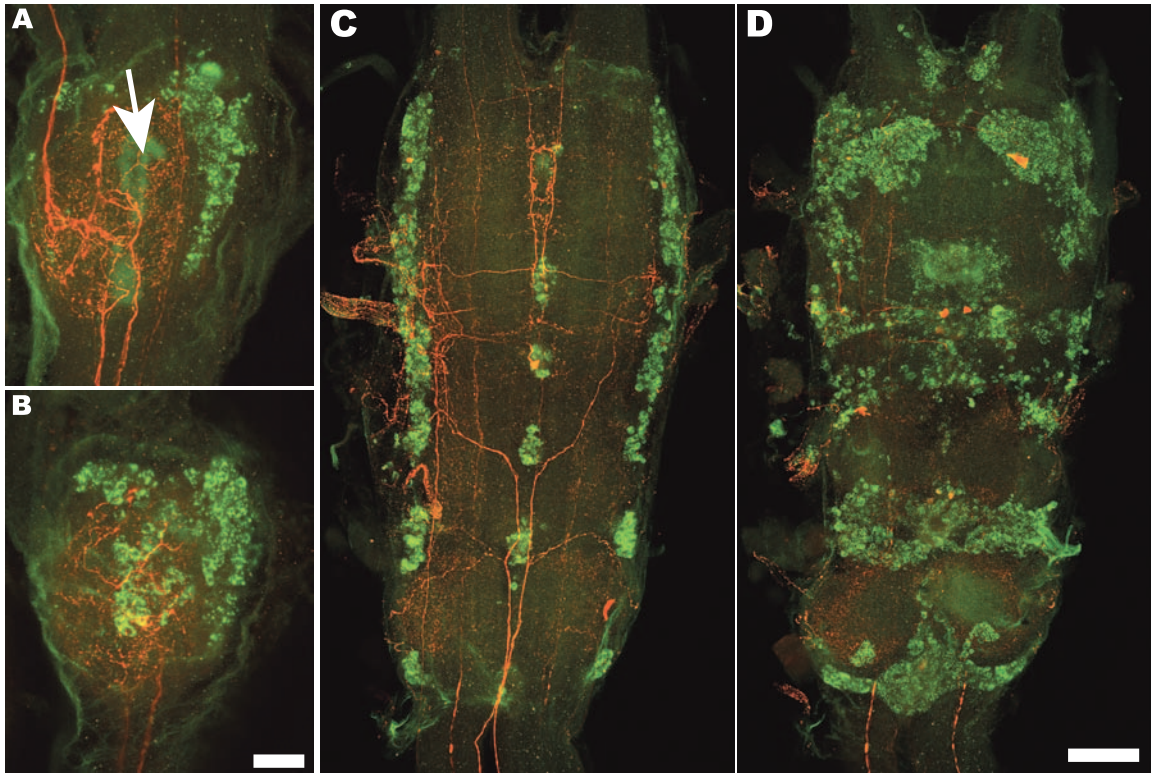
Figure 4-5: 5-HT$_{1\alpha crust}$ir (green) in the right circumesophageal ganglion (CEG, top) and in the subesophageal ganglion (SEG, bottom) with respect to 5-HTir (red). **A:** A stack of confocal slices spanning the dorsal half of the right CEG. In the center of the ganglion, an unidentified structure shows diffuse 5-HT$_{1\alpha crust}$ir (arrow). Additionally, the typical 5-HT$_{1\alpha crust}$ir punctate neuropil is seen in the core of the ganglion. **B:** A confocal stack of the ventral half of the CEG shows 5-HT$_{1\alpha crust}$ir somata throughout the cell body cluster at the lateral edge and the ventral surface of the ganglion. **C:** A collage of two confocal stacks spanning the dorsal half of SEG shows 5-HT$_{1\alpha crust}$ir somata throughout cell clusters at the midline and lateral edges of the ganglion. Punctate staining on the nerve at the left side of the image can also be seen. **D:** The ventral half of the SEG contains 5-HT$_{1\alpha crust}$ somata in a typical pattern. Punctate 5-HT$_{1\alpha crust}$ staining occurs in the neuropil cores of the ganglion but is difficult to see in overviews because somata are so brightly stained. All panels have been digitally brightened. Scale bars = 25μm in A and B; 200μm in C and D.

may be responding to neurohormonal or paracrine 5-HT signals while their dendrites may receive synaptic 5-HT input in the neuropil.

Thorax

5-HT$_{1\alpha crust}$ir in the thoracic ganglia has been previously described (Sosa et al., 2004). Briefly, the typical pattern of 5-HT$_{1\alpha crust}$ir shows label in a subpopulation of somata throughout all ganglia (Elson, 1996). 5-HT$_{1\alpha crust}$ir cells are arranged in a typical pattern along the midline and spreading to the lateral anterior and posterior corners on the ventral side of the ganglia. The lateral cell clusters wrap around the neuropil cores to the dorsal surface of the ganglion (Figure 4-6A-D). The appearance of these cells closely resembles that of the 5-HT$_{1\alpha crust}$ir somata in clusters 10 and 9&11 of the central brain (Figure 4-3B). In some preparations, somata of some 5-HTir cells in T1-4 but not T5 are also 5-HT$_{1\alpha crust}$ir (Figure 4-7A-C), indicating that 5-HT release from these cells may be regulated by 5-HT levels in the nerve cord. Thoracic 5-HT$_{1\alpha crust}$ir cells are not usually stained by backfilling nerves 1 or 2 of the ganglia (data not shown) indicating that most 5-HT$_{1\alpha crust}$ir cells are not motorneurons. The neuropil cores of the thoracic ganglia show 5-HT$_{1\alpha crust}$ir both alone and superposed with 5-HTir (Figure 4-7 D-F) in puncta along short stretches of neurites. While 5-HT$_{1\alpha crust}$ir is common in neuropil throughout the nerve cord, it is difficult to see in overview pictures because the stained somata are so bright.

Nerves 1 and 2 of the thoracic ganglia contain a plexus of 5-HTir varicosities that are thought to function as neurosecretory organs (Beltz and Kravitz, 1983; Real and Czternasty, 1990). In the crayfish this plexus is associated with similar patterns of 5-HT$_{1\alpha crust}$ir on both nerves. Most varicosities on nerve 2 show superposed 5-HTir and 5-HT$_{1\alpha crust}$ir (yellow) whereas others label only for 5-HT$_{1\alpha crust}$ (green) or the 5-HT (red) (Figure 4-8A-C). Often, 5-HT$_{1\alpha crust}$ir cell bodies are observed in the first and second
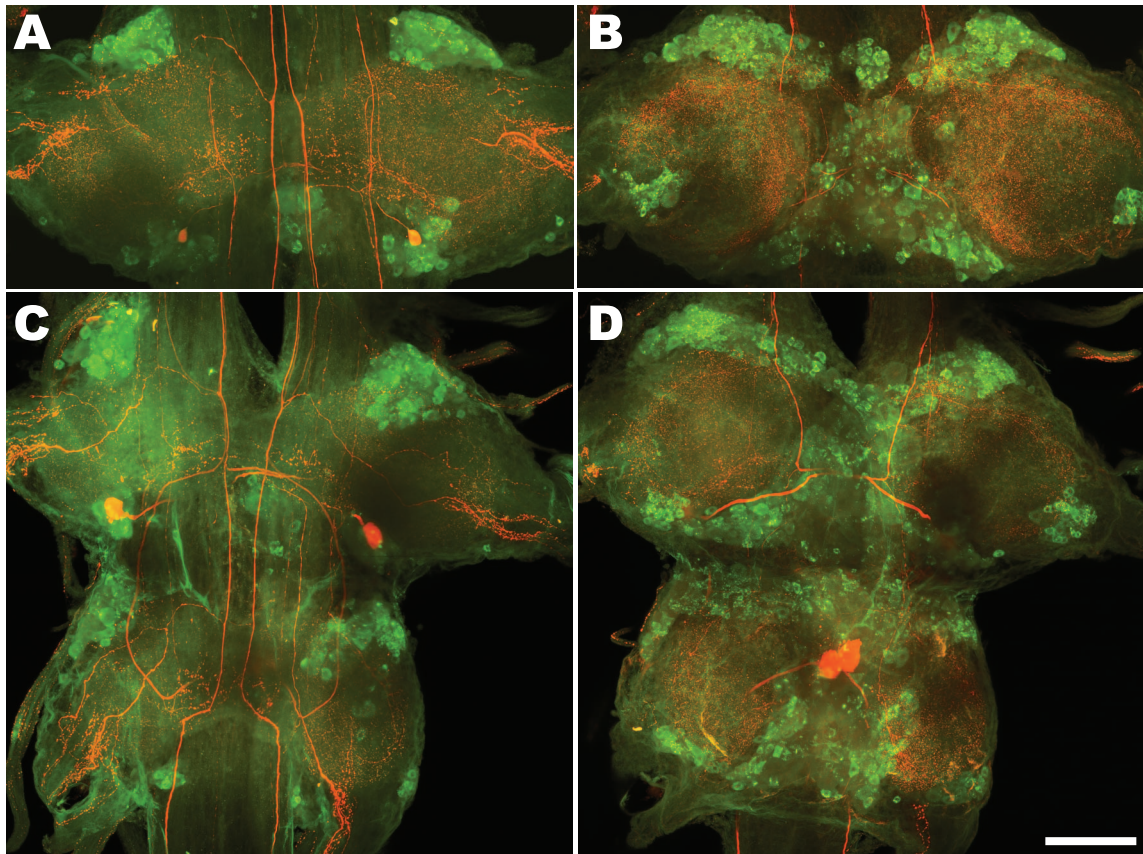
Figure 4-6: Thoracic ganglia display the typical pattern of 5-HT$_{1\alpha crust}$ir (green) distribution observed in ganglia throughout the nerve cord. Subsets of somata clusters along the midline and spreading to the lateral anterior and posterior corners on the ventral side of the ganglia are 5-HT$_{1\alpha crust}$ir with the lateral cell clusters wrapping around the neuropil cores to the dorsal surface of the ganglion. The various neuropils show a uniform distribution of 5-HT$_{1\alpha crust}$ir in all ganglia. 5-HTir (red) is also shown. **A:** A collage of two confocal stacks spanning the dorsal half of the third thoracic ganglion shows 5-HT$_{1\alpha crust}$ir somata in cell clusters at the anterior and posterior lateral corners of the ganglion. **B:** The ventral half of the ganglion shows that these lateral clusters of 5-HT$_{1\alpha crust}$ir somata are contiguous with clusters on the ventral side that fan out from a group of 5-HT$_{1\alpha crust}$ir neurons along the midline. **C:** A collage of four confocal stacks spanning the dorsal half of thoracic ganglia four and five show similar lateral clusters in these ganglia. **D:** The ventral half of these ganglia shows 5-HT$_{1\alpha crust}$ir somata distributed in the same typical pattern. The fluorescence has been digitally brightened in all of these panels and several pieces of surface debris have been digitally removed. Scale bars = 200μm.
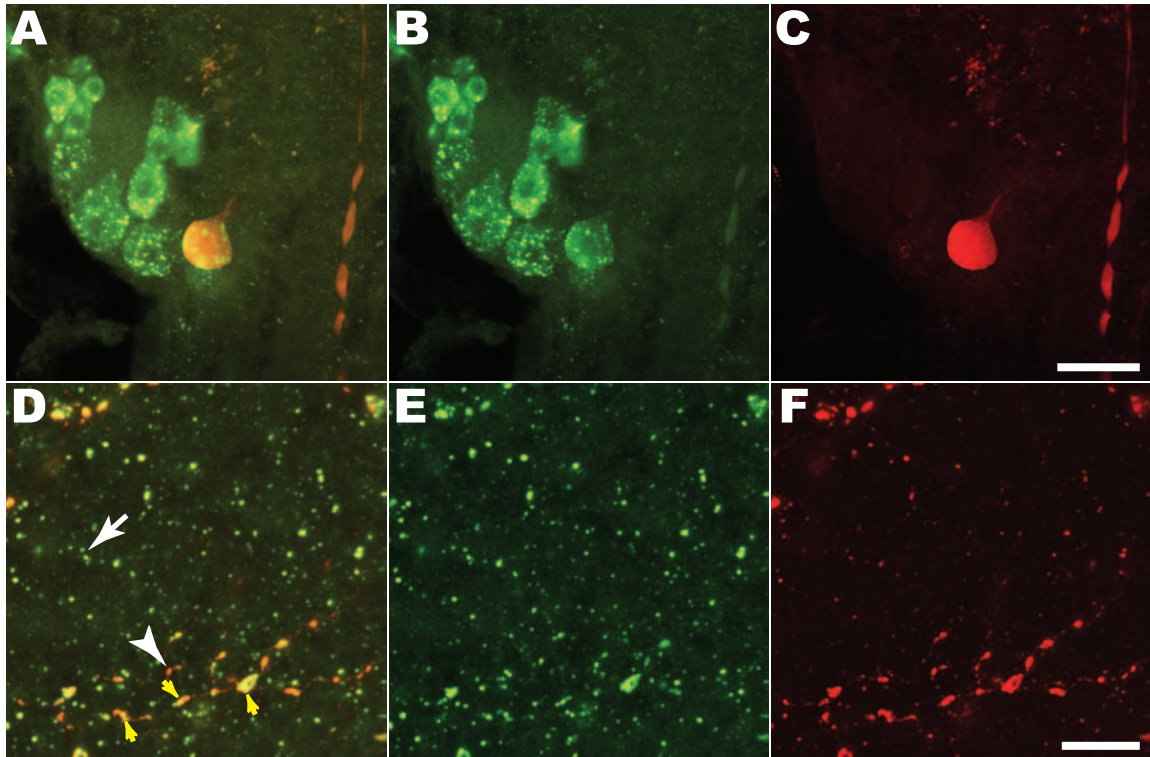
Figure 4-7: Somata of 5-HTir cells in thoracic ganglia 1-4 but not T5 or A1 sometimes show 5-HT$_{1\alpha crust}$ir. Neuropil in thoracic ganglia and throughout the nerve cord shows processes that label for transmitter or receptor alone or in superposition (yellow, yellow arrows). **A:** A stack of three confocal slices ~2 µm thick representing a thickness of ~10µm double-labeled for 5-HT (red, arrowhead) and 5-HT$_{1\alpha crust}$ (green, arrow). The receptor immunostaining appears as puncta in the cytoplasm and around the periphery of the 5-HT cell soma. **B:** The same image showing 5-HT label only. **C:** The same image showing 5-HT$_{1\alpha crust}$ label only. **D:** A stack of four confocal slices, spanning a thickness of ~4µm, showing neuropil in the core of a thoracic ganglion. Some processes label for 5-HT only (red) or for 5-HT$_{1\alpha crust}$ only (green) while others show superposition of transmitter and receptor (yellow). **E:** Same image showing only 5-HT label. **F:** Same image showing only 5-HT$_{1\alpha crust}$ label. The brightness of all panels has been digitally enhanced. Scale bars = 50µm for A-C and 10µm for D-F.
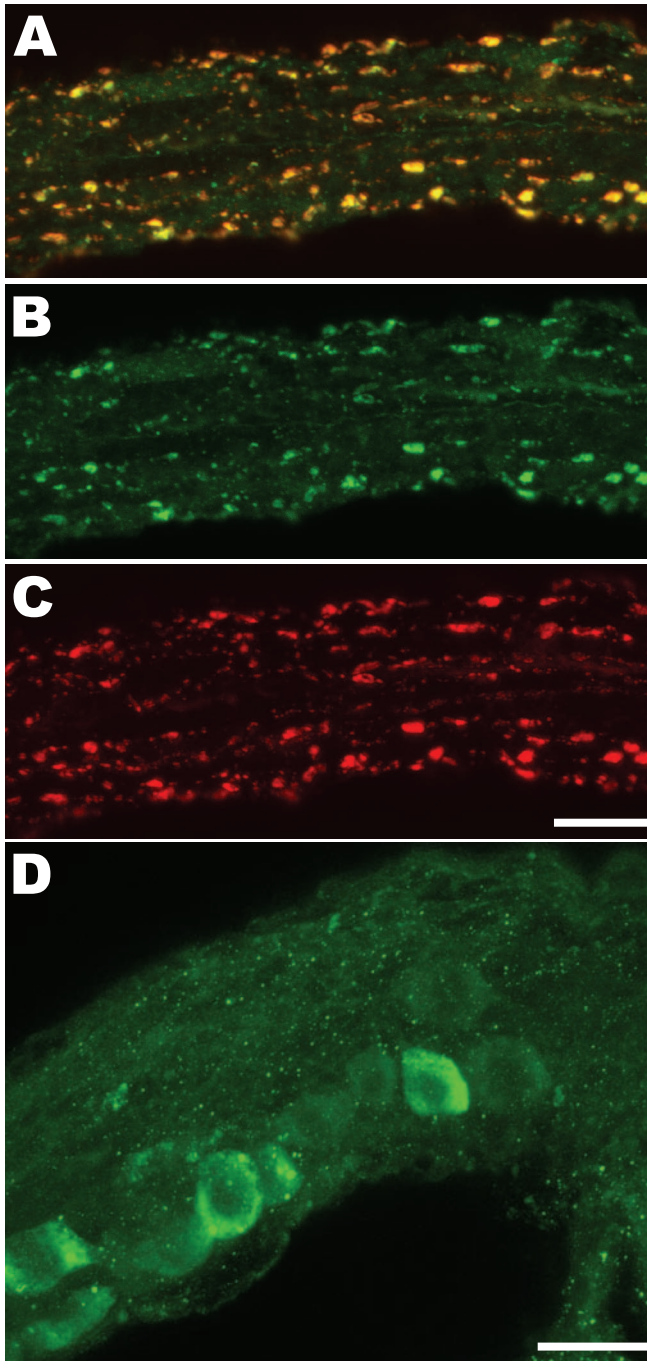
Figure 4-8: 5-HT$_{1\alpha crust}$ir (green) in a neural plexus on thoracic nerves with respect to 5-HTir (red). **A:** Stack of a few confocal slices showing the surface of nerve 2 from thoracic ganglion 2. Most of the 5-HT label is superposed with 5-HT$_{1\alpha crust}$ir (yellow). In a few locations transmitter or receptor occur alone. **B:** The same image showing only 5-HT. **C:** The same image showing only 5-HT$_{1\alpha crust}$. Careful comparison of B and C shows sites where labeling is stronger in B and other sites where fluorescence is stronger in C indicating that the apparent superposition is not due to bleed-through between channels during confocal imaging. **D:** 5-HT$_{1\alpha crust}$ir somata are common in the anterior branch of nerve 1 of thoracic ganglia. The posterior branch also often has 5-HT$_{1\alpha crust}$ somata but these number fewer than those in the anterior branch. All panels have been digitally brightened. Scale bars = 10µm for A-C, 25µm for D.

nerves of thoracic ganglia outside of the ganglion (Figure 4-8D). The number of 5-HT$_{1\alpha crust}$ir somata we have observed in nerve 1 in any one preparation ranges from 0 to 24 with most of these being restricted to the proximal section of the anterior branch of nerve 1. The second thoracic nerves generally contain fewer (0-5) 5-HT$_{1\alpha crust}$ ir somata. The number of nerve somata labeling is not constant from one thoracic ganglion to the other or even from one side of a ganglion to the other within individuals. More caudal thoracic ganglia tend to have a greater number of 5-HT$_{1\alpha crust}$ir cell bodies in their nerves.

Abdomen

5-HT$_{1\alpha crust}$ir is found on somata throughout the abdominal ganglia in a pattern similar to that seen in the thoracic ganglia (Figure 4-9A-F). The somata show irregular punctate staining for 5-HT$_{1\alpha crust}$ in the cytoplasm and around the periphery of the cell. Clusters of 5-HT$_{1\alpha crust}$ir cells are located along the ventral midline of the ganglion and sweep out to the lateral corners where they expand to the dorsal surface. As in T5, the large 5-HTir cells in A1 never colabel for 5-HT$_{1\alpha crust}$. A large, medial, usually unpaired cell is often 5-HT$_{1\alpha crust}$ir in ganglia A2-5 (Figure 4-9D, arrow). The terminal ganglion shows similar 5-HT$_{1\alpha crust}$ir patterns to the other abdominal ganglia; here the pattern of labeled somata underlines the fused nature of this ganglion (Kondoh and Hisada, 1983, 1986) (Figure 4-9F). Very occasionally a swimmeret motor neuron will be 5-HT$_{1\alpha crust}$ir but the great majority of 5-HT$_{1\alpha crust}$ir cells do not backfill from abdominal nerves (data not shown) indicating that 5-HT$_{1\alpha crust}$ is not primarily expressed by motorneurons. Neither the thin 5-HT fiber that follows the lateral giant neuron (Yeh et al., 1997) nor the lateral giant itself were found associated with 5-HT$_{1\alpha crust}$ir (not shown). The neuropil cores of abdominal ganglia contain both separate and superposed staining for 5-HT$_{1\alpha crust}$ir and 5-HTir similar to that seen in thoracic ganglia neuropil. 5-HT$_{1\alpha crust}$ir is uniformly distributed throughout the various neuropils of abdominal ganglia and does not appear
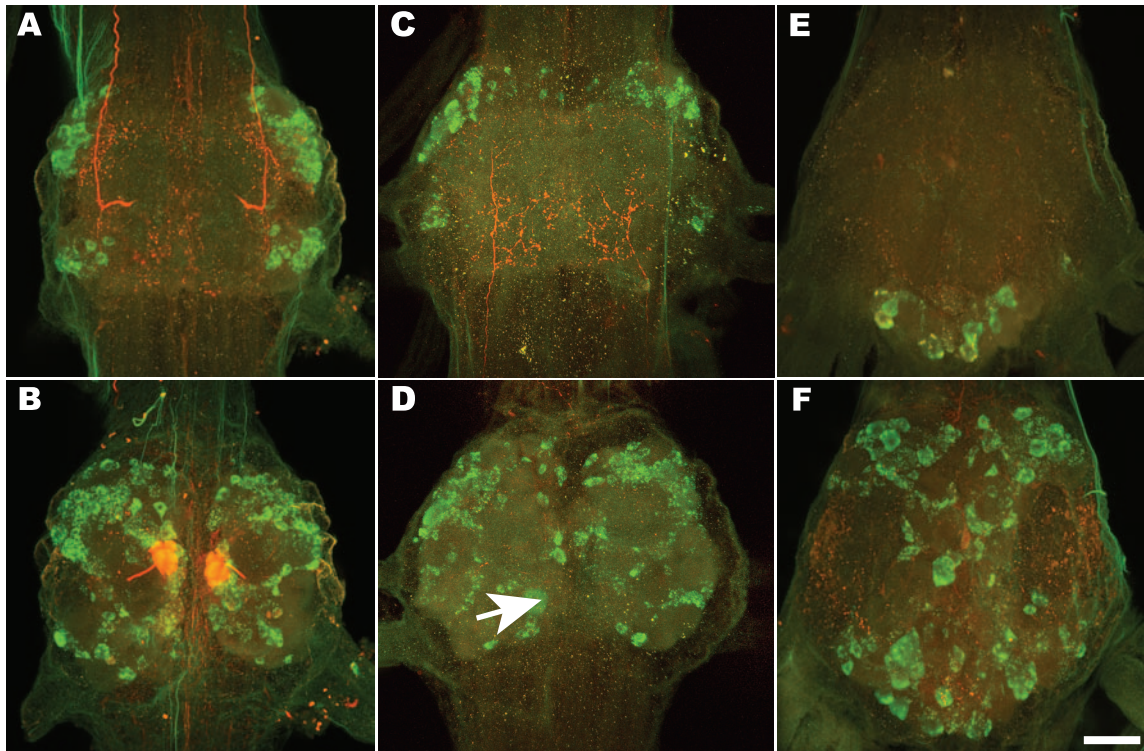
Figure 4-9: 5-HT$_{1\alpha crust}$ir (green) and 5-HTir (red) in abdominal ganglia. **A:** A stack of confocal slices comprising the dorsal half of A1 shows 5-HT$_{1\alpha crust}$ir somata at the lateral corners of the ganglion. **B:** The ventral half of A1 shows that the pattern of 5-HT$_{1\alpha crust}$ir somata in abdominal ganglia is reminiscent of that seen in thoracic ganglia. The large 5-HTir cells in A1 do not label for 5-HT$_{1\alpha crust}$ (yellow spots in this figure are an artifact of collapsing different cells that are separated on the dorsal-ventral axis on top of each other). **C:** A confocal stack of the dorsal half of A3 shows that the typical pattern of 5-HT$_{1\alpha crust}$ir somata distribution is maintained in all abdominal ganglia. **D:** The ventral half of A3 shows many 5-HT$_{1\alpha crust}$ somata one of which is a large unpaired medial neuron (arrow). This cell is commonly seen close to the midline on either side of abdominal ganglia 2-5. **E:** The dorsal half of the terminal abdominal ganglion, A6, shows 5-HT$_{1\alpha crust}$ir somata at the lateral edges of the final fused segment of the ganglion. **F:** The ventral half of A6 shows that the typical pattern of 5-HT$_{1\alpha crust}$ somata is maintained in each segment of this evolutionarily fused ganglion. In all ganglia, neuropil cores label for 5-HT$_{1\alpha crust}$ in a punctate pattern similar to that seen in neuropil of thoracic ganglia. The fluorescence has been digitally brightened in all panels. Scale bars = 100μm.

to be specified to identifiable neuropil areas. Cell fills of motor and sensory neurons (not shown) indicated that 5-HT$_{1\alpha crust}$ir is located in neuropils containing both sensory projections and motor dendrites and may be located on interneurons involved in global modulation of sensori-motor function. 5-HT$_{1\alpha crust}$ir somata of presumed sensory or neurosecretory neurons are often observed in nerves 1 and 2 of all abdominal ganglia (Figure 4-10) and in nerves 3 and 6 of A6. These somata stain in the typical punctate fashion and may appear alone or in small groups. They may be irregularly shaped like the cells inside the ganglia or spindle-shaped lying along the long axis of the nerve. Spindle-shaped 5-HT$_{1\alpha crust}$ir somata are more often seen far away from the ganglion. 5-HT$_{1\alpha crust}$ir cells in abdominal nerves are sometimes observed very distal from the ganglion, close to the body wall. All abdominal ganglion nerves have punctate 5-HT$_{1\alpha crust}$ir neuropil-like staining that occasionally delineates short stretches of neurite.

The superficial branch of nerve 3 of A1-5 carries processes that are 5-HT$_{1\alpha crust}$ir for short stretches in some preparations. This nerve contains motorneurons that innervate the abdominal superficial flexor muscles and thereby contribute to maintenance of abdominal posture (Fields et al., 1967). In some preparations the 5-HT$_{1\alpha crust}$ir fibers can be followed as they pass over the superficial flexor muscle for short distances (not shown). The superficial flexor muscle fibers themselves also express 5-HT$_{1\alpha crust}$ir in processes and patches on their surface (Figure 4-11). Neurohormonal 5-HT may therefore directly modulate abdominal posture via 5-HT$_{1\alpha crust}$ receptors on the superficial flexor muscles.

Vasculature

The surface of the ventral and sternal arteries of the circulatory system are surrounded by 5-HT$_{1\alpha crust}$ir projections (Figure 4-12 arrows). 5-HT$_{1\alpha crust}$ir fibers can be followed as they project along the arteries and 5-HT$_{1\alpha crust}$ir puncta are seen on the surface of the arteries. Staining for 5-HT$_{1\alpha crust}$ here does not appear to be associated with 5-HTir
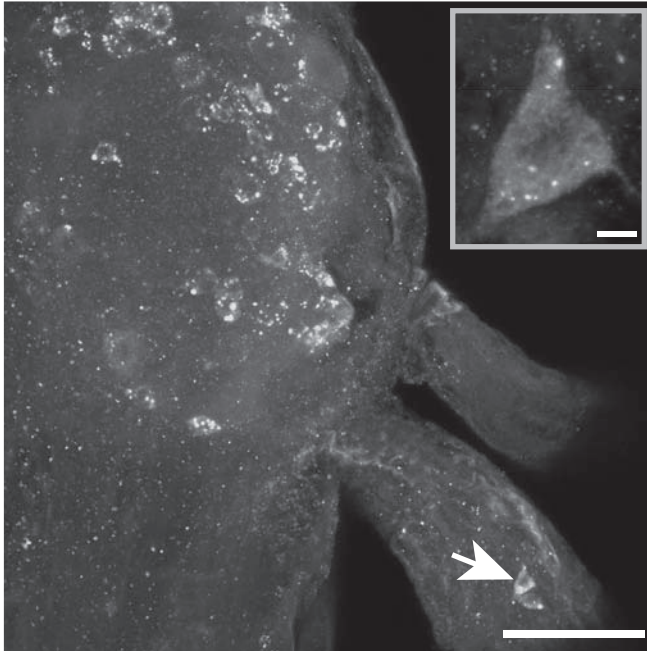
Figure 4-10: Somata of sensory or neurosecretory neurons in abdominal ganglia nerves 1 and 2 show 5-HT$_{1\alpha crust}$ir. These cells can be multipolar in shape (inset) or spindle-shaped (not shown). 5-HT$_{1\alpha crust}$ir cells may be found close to the ganglion or far out in the periphery close to the body wall. The image has been digitally brightened. Scale bars = 100μm, 5μm in inset.
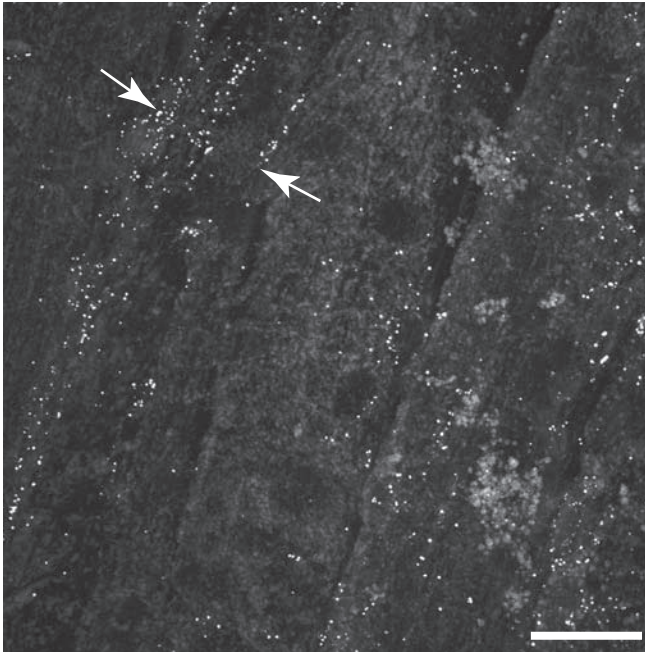
Figure 4-11: 5-HT$_{1\alpha crust}$ir is located in puncta along fibers and in patches on abdominal superficial flexor muscles. Scale bar = 25µm.
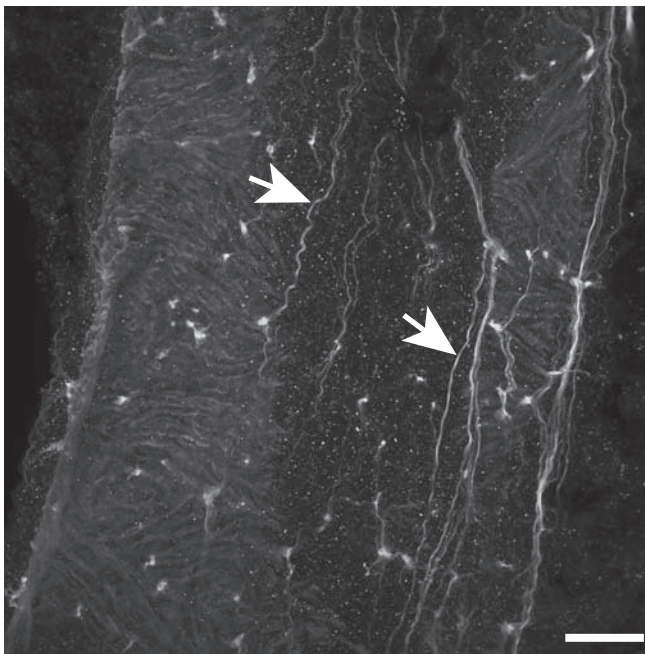
Figure 4-12: 5-HT$_{1\alpha crust}$ is expressed in puncta and along short stretches of neurites on the ventral artery. Similar staining is seen on the sternal artery. It is unclear whether these processes are located on a specific surface of the vasculature. Scale bar = 25µm.

(not shown). This pattern of staining is very distinct from nonspecific fluorescence sometimes observed along folds and creases in connective tissue. It is not clear whether the 5-HT$_{1\alpha crust}$ir fibers are localized to the inside, outside or both surfaces of the arteries. These fibers may represent neurons that sense 5-HT levels in the blood and exert general modulatory effects on the nervous system or on hemolymph flow levels in the arteries.

Variability in 5-HT$_{1\alpha crust}$ immunoreactivity

5-HT$_{1\alpha crust}$ expression is highly variable among animals but consistent within a given nervous system. Extensive label preparations show bright staining in the patterns described above with dozens of 5-HT$_{1\alpha crust}$ir somata in every cell cluster of the each ganglion. Limited label preparations show similar patterns with only a few equally brightly stained cells per cluster. Many nerve cords show a level of staining that is intermediate between extensive and limited levels of 5-HT$_{1\alpha crust}$ir, indicating that variability in 5-HT$_{1\alpha crust}$ir may represent a range of physiological states instead of an on/off mode of gene expression. In limited label preparations, thoracic ganglia show only a handful of 5-HT$_{1\alpha crust}$ir cells within the lateral clusters (Figure 4-13A). Because of this variability, approximation of the proportion of 5-HT$_{1\alpha crust}$ir cells in a given cluster is difficult. In abdominal ganglia of limited label nerve cords very few cells show 5-HT$_{1\alpha crust}$ir but one of these is commonly the large medial unpaired 5-HT$_{1\alpha crust}$ir cell in one or more abdominal ganglia (Figure 4-9D, arrow). This diversity in abundance of 5-HT$_{1\alpha crust}$ir seems to affect primarily somata as staining in the neuropil and on nerves appears at similar intensity and distribution in all preparations. The non-punctate 5-HT$_{1\alpha crust}$ir structure in the center of the circumesophageal ganglia is always present, even in low-level preparations but not in preabsorption controls. The level of staining does not correlate with the animal's gender, molt status, size or gut content, nor with food type, season, amount of time spent in the lab or time of day. This does not appear to be
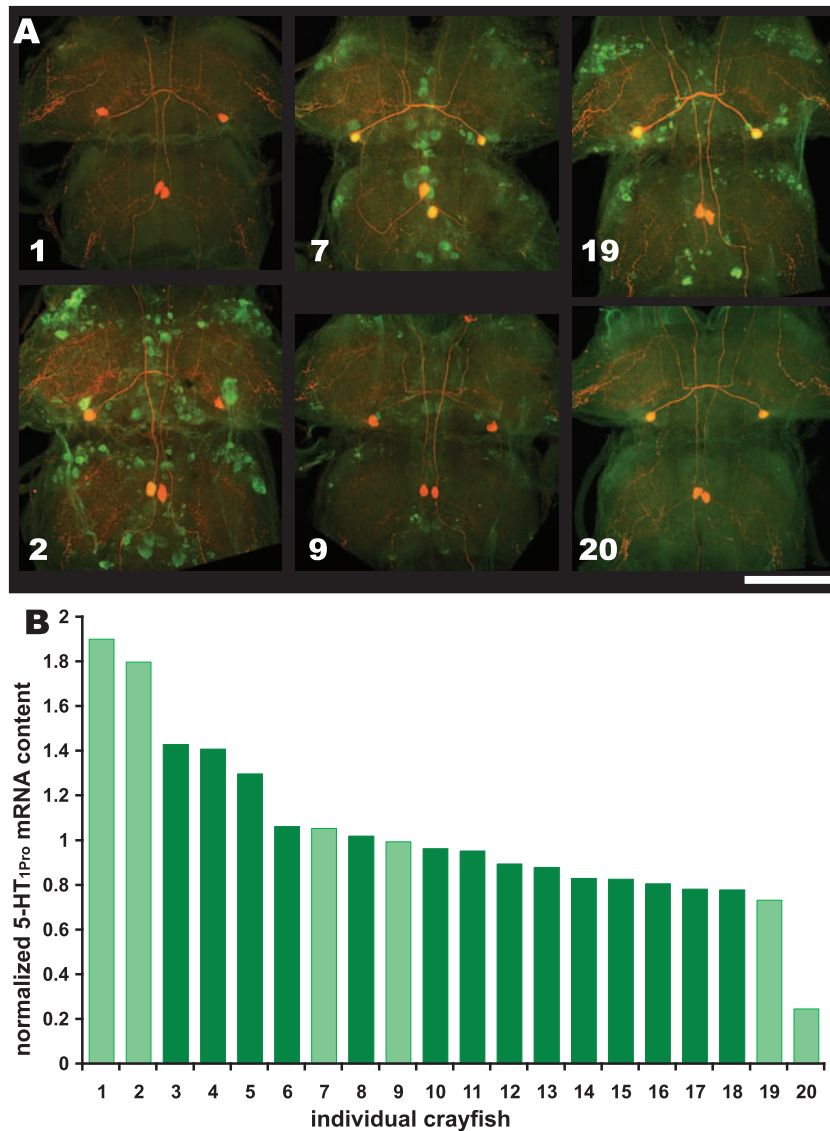
Figure 4-13: 5-HT$_{1\alpha crust}$ immunoreactivity and mRNA levels in the nervous system vary between animals. 5-HT$_{1\alpha crust}$ir levels do not directly correlate with relative levels of mRNA in an individual. **A:** 5-HT$_{1\alpha crust}$ir (green) on thoracic ganglia 4&5 from individual animals show that animals express variable levels of 5-HT$_{1\alpha crust}$ir. 5-HTir (red) is included as a control for immunocytochemistry technique. **B:** TaqMan quantitative RT-PCR of relative 5-HT$_{1\alpha Pro}$ mRNA levels in brains + CEG from the same animals as shown in A. Levels of receptor mRNA have been normalized to rRNA levels for each preparation. Comparison to 5-HT$_{1\alpha crust}$ir in the same individuals (light green bars) indicate that 5-HT$_{1\alpha crust}$ir levels do not necessarily correlate with mRNA levels. Individuals with high levels of 5-HT$_{1\alpha Pro}$ mRNA can show low (#1) or high (#2) levels of 5-HT$_{1\alpha crust}$ir. The same is true for nervous systems expressing medial (#7,9&19) and low (#20) levels of receptor mRNA. The fluorescence in A has been digitally brightened. Scale bar = 500μm.

an artifact of technique because the neuropil and the cells that do stain in limited-level nerve cords are just as bright and widespread through the nervous system as those in extensively labeled preparations as is 5-HTir (Figure 4-13A). In addition, preparations that were processed together, sometimes in the same tube, showed extensive and limited levels of 5-HT$_{1\alpha crust}$ir but equivalent 5-HTir, indicating that differential fixation or minor variations in protocol are probably not responsible for the variability.

Variability in 5-HT$_{1\alpha Pro}$ mRNA levels

The *Procambarus clarkii* sequence version of the 5-HT$_{1\alpha crust}$ receptor is called 5-HT$_{1\alpha Pro}$ and the real-time RT-PCR analysis of expression levels used primers and a probe specific for the crayfish mRNA sequence, therefore quantifying 5-HT$_{1\alpha Pro}$. Because the antibody used in immunocytochemistry was constructed to recognize the 5-HT$_{1\alpha}$ receptor in all three crustacean species, it is referred to as 5-HT$_{1\alpha crust}$ for immunocytochemistry data.

For this experiment, the brain and CEG of an individual were used to quantify 5-HT$_{1\alpha Pro}$ mRNA while thoracic ganglia T4&5 from the same animal were processed for 5-HT$_{1\alpha crust}$ir and 5-HTir. The variability in 5-HT$_{1\alpha crust}$ir may be partially due to differential levels of receptor expression because a similar variability is seen in 5-HT$_{1\alpha Pro}$ mRNA levels in individual nerve cords from isolate animals (Figure 4-13B). However, 5-HT$_{1\alpha Pro}$ mRNA levels do not correlate with the level of 5-HT$_{1\alpha crust}$ir observed in an individual nervous system (Figure 4-13). Nerve cords with extensive 5-HT$_{1\alpha crust}$ir may show high or medial levels of 5-HT$_{1\alpha Pro}$ mRNA (Figure 4-13: animals 2 & 19) while nerve cords with very limited 5-HT$_{1\alpha crust}$ir can contain high or low levels of 5-HT$_{1\alpha Pro}$ mRNA (Figure 4-13: animals 1 & 20).

Correlation of 5-HT$_{1\alpha Pro}$ mRNA levels with social status

Changes in social status of crayfish alter the modulatory effect of 5-HT on the responses of identified interneurons to sensory stimuli (Yeh et al., 1996; Yeh et al., 1997). Because these modulatory changes appear to result from corresponding changes in the balance of 5-HT receptors, we examined the effect of social status on 5-HT$_{1\alpha Pro}$ mRNA expression. Similar to earlier physiological experiments (Yeh et al., 1996; Yeh et al., 1997), juvenile crayfish were paired for two weeks, their relative status was monitored, and the levels of 5-HT$_{1\alpha Pro}$ mRNA in the nerve cord were measured in dominants and subordinates (Figure 4-14). In six of nine pairs tested, both animals had similar mRNA levels. However in three pairs, the dominant displayed a higher level than the subordinate; these three subordinates all had levels similar to the other six subordinates and their dominant partners. Consequently, although the levels of 5-HT$_{1\alpha Pro}$ mRNA across the nine animals did not significantly correlate with social status, the variability of 5-HT$_{1\alpha Pro}$ mRNA levels is significantly larger in dominant animals than subordinates (Figure 4-14, inset). In addition, whereas subordinate 5-HT$_{1\alpha Pro}$ mRNA levels are normally distributed, this is not true of levels in cords from dominant animals. This additional variability in dominant animals may reflect different degrees of social dominance.

Discussion

Molecular cloning and preliminary immunocytochemistry of the first crustacean type 1 serotonin receptor, 5-HT$_{1\alpha crust}$, was recently described by Sosa et al. (2004). When expressed in HEK cells, 5-HT$_{1\alpha crust}$ downregulates adenylate cyclase in response to 5-HT presumably via G$\alpha_{i/o}$-mediated pathways (Chapter 2). A detailed investigation of serotonin receptors in crustacea will greatly expand understanding of the cellular mechanisms underlying serotonergic modulation of circuits. These findings will be
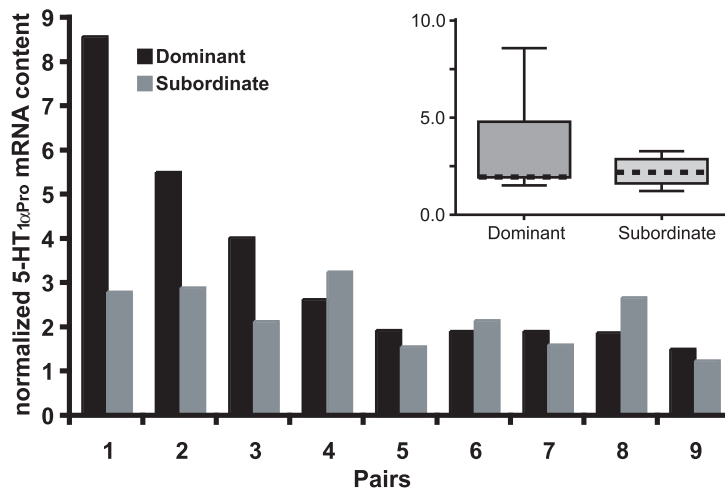
Figure 4-14: 5-HT$_{1\alpha Pro}$ mRNA variability in entire nerve cords from established dominant and subordinate animals does not correlate with social status (p=0.2, Wilcoxon Matched Pairs Test). However, receptor mRNA levels in dominants are not normally distributed and show significantly larger variance than subordinates (inset, p<0.01 for variances, F-test).

especially valuable because several crustacean circuits have been well developed as detailed model systems of serotonergic function.

Here we report the detailed immunocytochemical localization of $5\text{-HT}_{1\alpha crust}$ expression in the crayfish nerve cord, identifying possible sites of synaptic and neurohormonal action of 5-HT on the crayfish nervous system. While neuropil staining indicates that $5\text{-HT}_{1\alpha crust}$ may function as a synaptic receptor, strong $5\text{-HT}_{1\alpha crust}$ir also appears in somata throughout every ganglion. Although some of the punctate $5\text{-HT}_{1\alpha crust}$ir appears to be located around the periphery of the somata, a large proportion of puncta are localized to the cytoplasm possibly identifying structures of the endomembrane system. These structures may represent newly synthesized or recycled receptors. Similar punctate localization of serotonin and other G protein coupled receptors in somata has been previously reported in other nervous systems (Gerard et al., 1994; Ramaekers et al., 2001; Panek et al., 2003). Visualization of a D2-like dopamine receptor by fluorescence-coupled ligand binding showed that similar punctate labeling is localized to the surface of somata in cultured honeybee neurons (Kirchhof and Mercer, 1997). Immunocytochemistry with anti-synapsin has previously shown that clusters of somata in abdominal ganglia do not contain synapses (Mulloney and Hall, 2000) suggesting that any functional receptors expressed on these cell bodies may be responding to neurohormonal or paracrine serotonin signals. However, there is some evidence for synapses within soma clusters in the brains of crayfish and crabs (Sandeman et al., 1990). Most of the $5\text{-HT}_{1\alpha crust}$ir somata appear to be interneurons. Interneurons are modulated by serotonin in several crustacean circuits (Harris-Warrick, 1985; Teshiba et al., 2001; Nagayama, 2002), however, $5\text{-HT}_{1\alpha crust}$ir has not yet been associated with any of these identified interneurons. Expression of the $5\text{-HT}_{1\alpha crust}$ receptor on these neurons could result in a global regulation of circuit excitability in a manner analogous to that seen in mammalian brains. Approximately 10% of neurons in crayfish ganglia are γ-

aminobutyric acid (GABA)-immunoreactive neurons, most of which are interneurons (Mulloney and Hall, 1990). If these express 5-HT$_{1\alpha crust}$ they may function to mediate a global 5-HT signal by releasing GABA in a 5-HT-dependent manner. The source of neurohormonal serotonin in crustaceans is thought to be the pericardial organs (Sullivan, 1978) and the neurohormonal plexus of 5-HTir fibers on roots 1 and 2 of the thoracic ganglia (Livingstone et al., 1980; Beltz and Kravitz, 1983; Real and Czternasty, 1990); these roots also show a plexus of 5-HT$_{1\alpha crust}$ir.

Throughout the nervous system, 5-HT$_{1\alpha crust}$ir is localized to neuropil and to somata. Cytoplasmic staining may indicate newly synthesized receptor ready for shipping to the cell membrane or vesicles containing recycled receptor; while 5-HT$_{1\alpha crust}$ir puncta around the periphery of somata may represent functional aggregations of receptor. Localization to the cell body indicates that functional receptor here may be responsible for mediating signals that result in rapid changes in the transcriptional activity of the cell. In mice, 5-HT$_{1A}$ receptor activation has been implicated in modulating the level of transcription of certain clock genes in the suprachiasmatic nucleus during photic entrainment (Takahashi et al., 2002), the mechanism of this effect is not yet understood. In addition, 5-HT$_{1A}$ receptor activation in the rat brain regulates phosphorylation of mitogen-activated protein kinases (MAPKs), important signal transducers whose downstream targets include nuclear transcription factors (Chen et al., 2002).

Brain

Subpopulations of somata in all clusters, most prominently the 'globuli' cells of clusters 9&11 and 10, show strong labeling for 5-HT$_{1\alpha crust}$ in the crayfish brain. The local olfactory interneurons of clusters 9&11 and the olfactory projection neurons of cluster 10 are central olfactory pathway interneurons that have dendritic arborizations in glomeruli of the olfactory and accessory neuropils (Mellon et al., 1992). Pharmacological

depletion of 5-HT levels by injection with 5,7-dihydroxytryptamine (5,7-DHT) during development results in reduced and incorrect morphological arborization of the olfactory projection neurons (Sullivan et al., 2000). Proliferation and survival of these neurons during development is also reduced with 5,7-DHT treatment (Benton and Beltz, 2001). In decapod crustaceans neurogenesis in these clusters continues through adulthood (Schmidt, 1997; Schmidt and Harzsch, 1999) indicating that 5-HT$_{1\alpha crust}$ may be involved in transmitting 5-HT signals that shape the correct development and growth of the cells. The proportion and dispersed localization of 5-HT$_{1\alpha crust}$ir cells in clusters 9&11 and 10 is much greater than the population of proliferating cells (Schmidt and Harzsch, 1999) however, so 5-HT$_{1\alpha crust}$ could also be involved in mediating serotonergic modulation of higher level sensory processing by these cells (Mellon and Alones, 1995; Sandeman et al., 1995). Only a subset of somata located throughout the brain clusters express 5-HT$_{1\alpha crust}$. These may be representative of a specific class of interneuron; three different classes have previously been identified in cluster 11 based on odorant response patterns and morphology (Mellon and Alones, 1995). The 5-HT response of each of these classes of neurons has not been investigated. Some olfactory projection neurons are closely associated with tiny 5-HTir processes that project into the cluster from the lateral edges of the brain (Langworthy et al., 1997). A subpopulation of cluster 10 cells surrounded by 5-HTir neurites also show 5-HT$_{1\alpha crust}$ir with receptor and neurotransmitter superposed in puncta around the cell indicating a possible synaptic relationship; however this cannot be determined without electron microscopy. Because the receptor is more often unassociated with serotonergic projections and the projections that do exist are very small, the majority of 5-HT received by any functional 5-HT$_{1\alpha crust}$ receptors on these cells probably originates from neurohormonal signals. The brain receives controlled amounts of hemolymph via several arteries (Wilkens, 1999). The hemolymph may be the source of a general modulatory signal originating from the pericardial organ or second thoracic

roots that is targeted to clusters of 5-HT$_{1\alpha crust}$-expressing cells in the brain.

The dendrites of the olfactory projection neurons and local interneurons of clusters 10 and 9&11 innervate glomeruli of the olfactory and accessory lobe neuropils (Mellon et al., 1992). Within these glomeruli small processes stain for 5-HT$_{1\alpha crust}$ir, often appearing superposed with 5-HTir and sometimes alone; these latter may represent receptors responding to paracrine 5-HT signals. Not all 5-HTir processes superpose with 5-HT$_{1\alpha crust}$, indicating the possible presence of another type of 5-HT receptor, paracrine release or sections of processes that do not release neurotransmitter. The ultrastructural location of 5-HT$_{1\alpha crust}$ in these neuropils cannot be determined without electron microscopy, but it is likely that it is a post-synaptic receptor on olfactory interneurons. These receptors would receive synaptic 5-HT signals from a small group of 5-HT cells, including the dorsal giant interneuron (DGN), which innervate every glomerulus of the olfactory and accessory lobes (Sandeman et al., 1988). The DGN itself is not 5-HT$_{1\alpha crust}$ir on its soma or primary process, which supports the proposed postsynaptic role of 5-HT$_{1\alpha crust}$ in the glomeruli. While synapses on somata are rare in crustaceans, Substance P-immunoreactive processes have been shown to extensively invade and end in the lateral cell clusters in the brains of crayfish and crab. These processes make synapses primarily on the primary neurites of the somata but sometimes on the somata themselves or on a thin glial sheath around the cell bodies (Sandeman et al., 1990).

Other clusters in the brain also contain 5-HT$_{1\alpha crust}$ir somata. The function of the majority of these cells has not been determined but some neurons in these clusters have been identified as olfactory interneurons (Derby and Blaustein, 1988). These cells do not appear in close association with 5-HTir projections indicating that the 5-HT$_{1\alpha crust}$ receptors on their somata are most likely targeted by hormonal or paracrine 5-HT levels. Similarly, 5-HT$_{1A}$ somatodendritic autoreceptors in mammalian brains are also found in

extrasynaptic and nonsynaptic sites and are assumed to respond to diffuse 5-HT signals in the CNS (Riad et al., 2000).

Cells of the X-organ in the eyestalk ganglia of crustaceans produce and release crustacean hyperglycemic hormone (CHH) among other neuropeptides (Cooke and Sullivan, 1982). Serotonin-induced release of CHH in the eyestalk results in increased blood glucose levels and requires activation of 5-HT_1- and 5-HT_2-like receptors (Lee et al., 2000). Serotonergic excitation of X-organ cells and CHH release are also blocked by the serotonin antagonist methysergide (Sáenz et al., 1997; Lee et al., 2001) which has been identified as a specific antagonist of several invertebrate type 1 serotonin receptors (Tierney, 2001). The eyestalk is rich in serotonergic fibers and cells that release serotonin in an activity-dependent manner (Rodríguez-Sosa et al., 1997). These cells could modulate the neurohormonal state of the animal by differentially activating the many $5\text{-HT}_{1\alpha crust}$-expressing cells in the eyestalk. Serotonin in the eyestalk is also important for establishment of circadian rhythmicity in retinal sensitivity to light during development (Castanon-Cervantes et al., 1999; Escamilla-Chimal et al., 2001). The $5\text{-HT}_{1\alpha crust}$ receptor may therefore act to maintain circadian patterning in the adult via hormonal modulation of eyestalk cells by serotonin. Alternatively, localization of $5\text{-HT}_{1\alpha crust}$ to eyestalk cells may indicate modulation of visual sensitivity by hormonal or paracrine 5-HT levels.

Circumesophageal and Subesophageal Ganglia

The circumesophageal (or commissural) ganglion (CEG) is a part of a well-studied central pattern generating network controlling the movement of the foregut in crustacea, the stomatogastric nervous system. Cells in the CEG are important for coordination of foregut motor patterns (Selverston et al., 1976) and several somata in the CEG project to the stomatogastric ganglion (STG) to release modulatory substances, including 5-HT (reviewed in Harris-Warrick et al., 1992b). Neuropil superposition of 5-

$HT_{1\alpha crust}$ and 5-HT in the CEG indicates a possible synaptic pathway of modulation. The modulatory effects of serotonin on STG function have been examined at the cellular level in the STG but not the CEG (reviewed in Harris-Warrick et al., 1992a; Nusbaum and Beenhakker, 2002). In the STG of the spiny lobster, $5\text{-}HT_{1\alpha crust}$ is expressed in somata of stomatogastric neurons (Clark and Baro, 2003).

Neurons in the subesophageal ganglion (SEG) are responsible for innervation and coordination of mouthparts. In addition to this local function, the SEG also plays an important role in regulating the motor activity of the entire animal (reviewed in Altman and Kien, 1987). The repeated pattern of stained somata in this ganglion is very much like that observed in the thoracic ganglia (Sosa et al., 2004) and underlines the evolutionarily fused nature of the SEG. Mediation of a hormonal 5-HT signal here via $5\text{-}HT_{1\alpha crust}$ on local and projecting interneurons may function to modulate the activities of the mouthparts and behavior of the animal.

Thorax

In thoracic ganglia, $5\text{-}HT_{1\alpha crust}$ is expressed on somata in a typical pattern (Sosa et al., 2004) with stained somata lying along the ventral midline and fanning out to anterior and posterior lateral clusters. These lateral clusters expand to the dorsal surface and contain $5\text{-}HT_{1\alpha crust}$ir cells throughout. In thoracic ganglia 1-4 the posterior lateral clusters contain the somata of 5-HTir cells (Beltz and Kravitz, 1983; Real and Czternasty, 1990; Antonsen and Paul, 2001), which sometimes stain for $5\text{-}HT_{1\alpha crust}$ir. This indicates that these serotonergic cells may themselves be regulated by 5-HT. We hypothesize that most of the $5\text{-}HT_{1\alpha crust}$ir cells observed within the thoracic ganglia are interneurons as we have been largely unable to backfill any $5\text{-}HT_{1\alpha crust}$ir somata from peripheral motor nerves. The walking system of crayfish is modulated by 5-HT via direct and polysynaptic pathways (Rossi-Durand, 1993; Pearlstein et al., 1998; Le Bon-Jego et al., 2004) which

may involve some of the 5-HT$_{1\alpha crust}$ir cells identified here. Thoracic nerve 1 often contains 5-HT$_{1\alpha crust}$ir somata which are similar in appearance and location as previously described sensory neurons including mechanoreceptors that process information from varied receptor fields (Bevengut et al., 1983). Similar somata are observed less frequently and in lower numbers in nerve 2. Cells with somata here are also thought to be neurosecretory cells and a subset of these has been shown to contain and release the neuropeptide crustacean hyperglycemic hormone (CHH) upon stimulation (Chang et al., 1999) in the lobster. These sensory neurons may be subject to paracrine or hormonal serotonergic modulation. Peripheral modulation of a sensory stretch receptor by 5-HT has been shown in the lobster; this is thought to occur in response to neurohormonal 5-HT signals (Pasztor and Bush, 1987). Serotonin has also been shown to inhibit the firing of somata in thoracic nerve 2 (Konishi and Kravitz, 1978), an effect that may be mediated by 5-HT$_{1\alpha crust}$.

The 5-HTir neuropil in the core of each thoracic hemiganglion is regularly seen associated with 5-HT$_{1\alpha crust}$ir. Some fibers in the neuropil show superposed label for the neurotransmitter and receptor, suggesting a synaptic interaction, while others label for only one or the other. This may indicate that other classes of serotonin receptors are also expressed in these areas and that 5-HT$_{1\alpha crust}$ on some fibers may be responding to paracrine or hormonal levels of 5-HT.

Nerves 1 and 2 of the thoracic ganglia have strongly 5-HTir plexuses of fibers that originate in the ganglion and fan out on the surface of the nerves (Beltz and Kravitz, 1983; Real and Czternasty, 1990; Antonsen and Paul, 2001). A similar plexus is seen to label for 5-HT$_{1\alpha crust}$ir. Most processes show superposed label for transmitter and receptor indicating a possible synaptic or autoreceptor role for 5-HT$_{1\alpha crust}$ on modulatory interneurons. The 5-HTir plexus is thought to have neurohormonal functions (Beltz and Kravitz, 1983) and 5-HT$_{1\alpha crust}$ir that is not associated with 5-HTir here may be functioning

as a general modulatory receptor on interneurons responding to paracrine 5-HT signals. The spike initiation zones of motor neurons are thought to occur in the proximal portion of their axons, in the vicinity of the nerve roots (Evoy, 1977). 5-HT$_{1\alpha crust}$ir in the corresponding portions of thoracic nerve roots may indicate that these are sites where 5-HT indirectly modulates motor neuron excitability through global interneuron pathways.

Abdomen

Somata on the ventral surface of the abdominal ganglia label in the typical way for 5-HT$_{1\alpha crust}$ir. These somata are arranged in a pattern that is reminiscent of that observed in thoracic ganglia. Midline somata labeled for 5-HT$_{1\alpha crust}$ are larger and often include a very prominent, usually unpaired large cell located medially and usually slightly posterior. As in thoracic ganglia, ventral 5-HT$_{1\alpha crust}$ir cell body clusters expand to the dorsal surface at the lateral anterior and posterior edges of the abdominal ganglia. These somata probably belong to interneurons as they are not stained by backfill of the motor nerves. Very occasionally a single swimmeret motorneuron will label for 5-HT$_{1\alpha crust}$ir. Application of 5-HT results in an initial facilitation of swimmeret motor nerve activity followed by termination of the activity (Barthe et al., 1993). These effects resulted from 5-HT application to the thorax indicating that the effects could be mediated by inhibitory interneurons that could express 5-HT$_{1\alpha crust}$. Nagayama et al. (1997; 2002) showed that a population of nonspiking local interneurons in the terminal abdominal ganglion (A6) is hyperpolarized by bath application of serotonin. This interneuronal inhibition results in a decrease in spiking frequency of the exopodite reductor motor neuron. Expression of 5-HT$_{1\alpha crust}$ by these interneurons could mediate this effect. Serotonin is also an important modulator of the lateral giant (LG) interneuron, which is a command neuron for a stereotyped tailflip escape behavior (reviewed in Edwards et al., 1999). Different serotonin superfusion doses, rates and durations elicit dual and

opposing effects in the LG, indicating that 5-HT activates two parallel signaling pathways in the LG (Teshiba et al., 2001). In addition, the effect of 5-HT on the LG's response to mechanosensory afferent input depends on the social status of the animal (Yeh et al., 1996; Yeh et al., 1997). While 5-HT superfusion results in facilitation of LG in dominants, the same treatment has an inhibitory effect in subordinate animals. Because LG does not appear to express 5-HT$_{1\alpha crust}$, any 5-HT modulation of the LG circuit via this receptor must occur at another point in the circuit, such as on interneurons that express 5-HT$_{1\alpha crust}$. Serotonin application modulates the efficacy of coupling between sensory afferents and LG (Antonsen, personal communication) providing a possible target for 5-HT$_{1\alpha crust}$-expressing modulatory interneurons. Alternatively, 5-HT$_{1\alpha crust}$ may not be one of the receptors involved in serotonergic modulation of the LG circuit.

Many preparations contain 5-HT$_{1\alpha crust}$ir cell bodies in nerves 1 and 2 of the abdominal ganglia, sometimes a considerable distance from the ganglion. Some of these cells are multipolar in shape and may be cutaneous mechanoreceptors (Pabst and Kennedy, 1967), others are spindle-shaped and may represent other types of sensory receptors or neurohormonal modulator cells as seen in the thoracic nerves. Expression of 5-HT$_{1\alpha crust}$ on these cells indicates that sensory processing may be modulated by hormonal levels of serotonin.

Serotonergic modulation via 5-HT$_{1\alpha crust}$ appears to occur in all abdominal ganglia neuropils as well, here some processes show superposed label for serotonin and 5-HT$_{1\alpha crust}$ while others label only for one or the other. Serotonergic effects on the abdomen of crustacea have been studied for decades; injection of serotonin into the hemolymph of crustacea results in a flexed abdomen and stereotyped posture (Livingstone et al., 1980). This posture has been associated with an increase in aggressive behavior in some studies (Kravitz, 1988; Antonsen and Paul, 1997; Huber et al., 1997a) but the opposite effect has also been found (Peeke et al., 2000; Tierney and Mangiamele, 2001). Tierney and

Mangiamele (2001) showed that some aspects of the '5-HT posture' could be elicited by injection of the vertebrate $5\text{-HT}_1/5\text{-HT}_5/5\text{-HT}_7$ agonist, 5-CT (5-carboxamidotryptamine maleate), which is active at some invertebrate 5-HT_1 receptors (Tierney, 2001) including $5\text{-HT}_{1\alpha crust}$ (Chapter 2). 5-CT may therefore activate the $5\text{-HT}_{1\alpha crust}$ receptor and contribute to some aspects of the '5-HT posture'.

Serotonin also affects abdominal posture by modulating the efficiency of the neuromuscular junction in crayfish. Application of 5-HT results in presynaptic enhancement of transmitter release from the motor neurons (Dudel, 1965; Livingstone et al., 1980; Fischer and Florey, 1983; Strawn et al., 2000) mediated by activation of a phosphatidylinositol (IP_3) messenger pathway (Dixon and Atwood, 1989). Because motor neurons do not appear to express $5\text{-HT}_{1\alpha crust}$, serotonergic modulation of these cells may occur through different classes of 5-HT receptors or via interneurons expressing $5\text{-HT}_{1\alpha crust}$. Serotonin application also results in excitation of crustacean muscles and stretch receptors directly (Pasztor and Golas, 1993; Strawn et al., 2000). The $5\text{-HT}_{1\alpha crust}$ receptor is expressed in patches and fibers on the surface of the superficial flexor muscles, which are important in maintenance of abdominal posture. Modulation of abdominal posture has long been proposed to occur via neurohormonal actions of 5-HT (Livingstone et al., 1980; Harris-Warrick and Kravitz, 1984; Strawn et al., 2000) and this widespread pattern of receptor expression on the muscles supports a proposed role of $5\text{-HT}_{1\alpha crust}$ in mediating general modulation of posture. $5\text{-HT}_{1\alpha crust}$ir located on neurons that project along the motor nerve and across the superficial flexor muscles provide a possible target for further neurohormonal or more specific synaptic modulation. In addition, Harris-Warrick (1985) showed serotonergic inhibition of command element interneurons of the postural system providing a possible central role for modulation via the $5\text{-HT}_{1\alpha crust}$ receptor on interneurons within the nerve cord.

Vasculature

In mammals, 5-HT receptors have been implicated in modulating blood pressure both centrally and peripherally. Pharmacological activation of central 5-HT$_{1A}$ receptors results in both sympatho-inhibition and, perhaps via an adrenaline pathway, –excitation. A rise in blood pressure is seen when 5-HT$_{1B}$ receptors are activated while activation of 5-HT$_{1D/1F}$ results in hypotension (Ramage, 2001). Peripherally, the 5-HT$_{1B}$ receptor on the surface of smooth muscle cells in pulmonary arteries acts as an important mediator of vasoconstriction in humans (reviewed in MacLean et al., 2000). In crustaceans, 5-HT perfusion increases heart rate and force (Florey and Rathmayer, 1978; Wilkens, 1999). In addition, resistance to fluid flow in the arteries is increased with 5-HT perfusion with the proposed site of 5-HT action being the muscular bicuspid valves at the origin of each artery (Wilkens, 1997). Unlike mammalian blood vessels, crustacean arteries, with the exception of the dorsal abdominal artery, do not have muscle layers (Martin and Hose, 1995; Wilkens et al., 1997) and are therefore not subject to direct vasoconstrictive modulation. The 5-HT$_{1\alpha crust}$ receptor observed on the surface of the vasculature could therefore be on processes that send signals to the valves to regulate hemolymph flow. Alternatively, processes expressing 5-HT$_{1\alpha crust}$ here may be monitoring hormonal levels of 5-HT in the hemolymph and sending this information back to the central nervous system to exert a general modulatory effect.

Variable levels of 5-HT$_{1\alpha crust}$ir

Expression of 5-HT$_{1\alpha crust}$ in the nerve cord of crayfish is highly variable between animals with a few preparations showing very high numbers of labeled somata, some showing only a handful and many appearing in some median state between these two extremes. A given nerve cord, however, will have a consistent level of 5-HT$_{1\alpha crust}$ir throughout; we have never observed a preparation with, for example, an extensively

labeled brain and a limited label abdomen. The somata that do show $5\text{-HT}_{1\alpha crust}$ir and neuropil in limited-label preparations are just as bright as those in extensive-label preparations. Differences in $5\text{-HT}_{1\alpha crust}$ir were not correlated with the animals' size, gender, molt status, gut contents nor with the time of day sacrificed or the length of time in the lab. Extensive and limited level preparations were obtained from two different animals when the nerve cords were processed together in the same tube, ruling out small procedural variations such as differential fixation. While these findings indicate that $5\text{-HT}_{1\alpha crust}$ir levels may be indicative of a physiological state of the animal, a more complex interaction of these and other factors may be responsible for the variability in immunoreactivity. Interestingly, most of the variation seen in $5\text{-HT}_{1\alpha crust}$ir is in staining of the somata as opposed to neuropil. This may indicate that the receptor is processed differently during synthesis or recycling by individual animals. Many studies have shown that serotonin levels and activity of 5-HT receptors in mammals are sensitive to stress, including social stress (reviewed in Chaouloff et al., 1999). Laaris et al. (1997) found that extended exposure to a novel environment resulted in decreased sensitivity of dorsal raphe nucleus 5-HT_{1A} receptors in the rat. Expression levels of the 5-HT_{1A} receptor in the hippocampus are also affected by chronic stress and vary with the intensity and predictability of the stressor (McKittrick et al., 1995; Lopez et al., 1998). Serotonin receptor activity is therefore sensitive to modulation by the behavioral state and experience of an animal. Some of the high degree of variability seen in the level of $5\text{-HT}_{1\alpha crust}$ir in the crayfish nerve cord may stem from transcriptional regulation as $5\text{-HT}_{1\alpha Pro}$ mRNA (the crayfish-specific version of $5\text{-HT}_{1\alpha crust}$) levels are also highly variable. However, the level of $5\text{-HT}_{1\alpha Pro}$ mRNA does not necessarily correlate with the level of $5\text{-HT}_{1\alpha crust}$ir seen in the same animal, indicating that post-transcriptional points of regulation may contribute to $5\text{-HT}_{1\alpha crust}$ expression. Alternatively, postranslational modifications such as glycosylation or oligomerization may alter antibody affinity

in immunocytochemistry resulting in the varied levels of 5-HT$_{1\alpha crust}$ir observed. Mammalian 5-HT receptors are regulated through alternative splicing, mRNA editing and oligomerization; such modifications can affect ligand-binding, G-protein coupling or tissue distribution of the receptor (reviewed in Kroeze et al., 2002) and may affect immunoreactivity resulting in differential levels of 5-HT$_{1\alpha crust}$ir detected.

Effect of social status on 5-HT$_{1\alpha Pro}$ mRNA levels

Changes in social dominance affect many aspects of crayfish behavior, including agonistic and non-agonistic behaviors (Kravitz, 1988; Herberholz et al., 2003) several of which are modulated by serotonin. For one cell, the LG command neuron for escape, the modulatory effect of serotonin is known to change with the animal's social status, becoming facilitatory in social dominants and inhibitory in subordinates (Yeh et al., 1996; Yeh et al., 1997). Changes in the facilitatory and inhibitory effects of 5-HT agonists parallel the changes of serotonin and suggest that the balance of facilitatory and inhibitory serotonin receptors changes with social status. The results presented here suggest that levels of 5-HT$_{1\alpha Pro}$ mRNA expression in some dominants are greater than those of their subordinate partners and than those of other dominant and subordinate pairs. We do not know why mRNA levels should come to vary more among dominants than among subordinates; but it is clear that expression levels of 5-HT$_{1\alpha Pro}$ are not directly correlated with gender, molt stage, size, feeding, length of time in the lab or the time of sacrifice in the animal's circadian cycle. However, a complex interaction of these or other factors may contribute to the variability in receptor expression. In these experiments, no measures of the relative degree of dominance were made; differences in the degree of dominance are apparent in the interactions of a pair of animals and can be significant (Issa et al., 1999). It may be that the larger range of mRNA variation seen

here in dominant crayfish is associated with a correspondingly larger range of socially
dominant behavior.

Conclusions

The type 1 serotonin receptor, 5-HT$_{1\alpha crust}$, is expressed throughout the nerve
cord of the crayfish in neuropil and somata. The cells expressing 5-HT$_{1\alpha crust}$ are
probably primarily interneurons that modulate various circuits in response to synaptic,
neurohormonal and paracrine 5-HT signals. 5-HT$_{1\alpha crust}$ is also expressed on nerve plexuses
and on muscle tissue implicating it further in neurohormonal signaling modulating broad-
scale effects such as posture. Molecular characterization of the 5-HT$_{1\alpha crust}$ receptor, its
pharmacology and second-messenger properties (Chapter 2) will allow studies of how
this molecule functions in the modulation of circuits and behavior.

Chapter 5 – General Discussion

Crustacean 5-HT systems exist in multiple states that change on variable time scales.

Obtaining state-appropriate responses from the same modulatory stimulus.

Crustacean 5-HT systems are plastic and exhibit different stable states that are maintained on different time scales. These physiological states reflect the necessity of responding appropriately to the same stimulus in different contexts. For example; the lateral giant (LG) escape circuit of crayfish is critical for eliciting escape behaviors in response to predation. During agonistic interactions, 5-HT may be released into bloodstream (Sneddon et al., 2000). Depending on the previous social experience of the animal, the modulatory effects of 5-HT on the LG response differ. While the escape response is facilitated in dominant animals, it is inhibited in subordinates (Yeh et al., 1996). This difference may reflect the distinct adaptive requirements of each animal; subordinates may have better success by responding with controlled swimming rather than with stereotyped escape maneuvers. Interestingly, state changes associated with social status occur on two time scales. Animals assume dominant or subordinate behaviors within minutes of engaging in agonistic interactions. The cellular 5-HT response, however, changes gradually over a period of two weeks (Yeh et al., 1996; Yeh et al., 1997). This indicates that multiple physiological mechanisms are susceptible to state-dependent alterations in the crayfish nervous system. In the spiny lobster we found that the pyloric circuit also exists in two states. It is as yet unclear how stable these states are or what experience or physiological factor they may be associated with.

The structure, signaling and pharmacology of crustacean serotonin receptors are evolutionarily conserved.

Conservation of 5-HT receptor structure in crustaceans.

The conservation of 5-HT receptor sequence and function through evolution speaks to the importance of serotonergic modulation in the nervous system. While overall sequence identity can drop quickly when comparing receptors from different species, the key residues and regions important to receptor function are faithfully conserved from nematodes up to humans. Predictably, therefore, in crustacean orthologs of $5\text{-HT}_{2\beta}$ and $5\text{-HT}_{1\alpha}$ receptors, important amino acids within the transmembrane domains that form the ligand binding pocket are highly conserved. In addition, regions at the intracellular membrane face that are important for signal transduction are conserved for each receptor subtype. Indeed, the core regions of $5\text{-HT}_{2\beta}$ and $5\text{-HT}_{1\alpha}$ are practically identical between crayfish and spiny lobster.

Signaling of crustacean $5\text{-HT}_{2\beta}$ and $5\text{-HT}_{1\alpha}$ receptors

Due to the conservation of key regions, 5-HT receptors of the same class use the same G protein effector to transduce their primary signals. For example, when expressed in HEK cell lines, 5-HT_1 receptors from diverse species couple to $G\alpha_{i/o}$ to effect an inhibition of adenylyl cyclase. Similarly, 5-HT_2 receptors activate phospholipase Cβ via $G\alpha_q$ (reviewed in Blenau and Baumann, 2001; Hoyer et al., 2002; Kroeze et al., 2002). Crustacean 5-HT receptors are no exception to this general rule, as expected based on the presence of conserved key residues and domains within the proteins. A rapidly expanding body of work, however, shows this generalization to be a gross simplification of 5-HT receptor signaling in actual physiological systems. First, the downstream effectors of the signaling cascades, including Gα subunits, can be differentially configured. Second, an activated G protein can transduce a signal not only through the Gα subunit, but also

couples to various second-messenger cascades via the membrane-bound G$\beta\gamma$ subunit (Raymond et al., 2001). The presence of multiple G protein isoforms adds a level of complexity to the immediate signaling process. Third, the 5-HT receptors themselves are variable due to mechanisms such as alternate splicing and RNA editing (Sanders-Bush et al., 2003). Fourth, receptors aggregate into oligomers with distinct functions (Lee, 2003). Finally, 5-HT receptors couple to a seemingly endless array of G protein independent signaling pathways mediated through a variety of interacting proteins (reviewed in Heuss and Gerber, 2000). Expression in heterologous systems and identification of ligand specificity and signaling pathways is important in characterizing a receptor and providing a 'handle' for its study *in vivo*. However, when these receptors are studied in their natural environment, these alternate factors must be kept in mind as possible mechanisms that may be acting to mediate the receptor's signal. Such properties may not always be observable or immediately obvious. Even in cell culture, secondary signaling effects and cell-specific properties can complicate characterization of a receptor (Clark and Baro, in review).

Pharmacology of crustacean 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ receptors is conserved.

It is clear that pharmacological properties of 5-HT receptors are not conserved between mammals and invertebrates (Colas et al., 1997; Blenau and Baumann, 2001; Tierney, 2001). Studies that have sought to dissect invertebrate 5-HT signaling mechanisms, using drugs characterized on mammalian receptors, have therefore been suggestive but limited as the pharmacological profiles of invertebrate receptors are highly variable among species (Colas et al., 1997; Tierney, 2001). Systematic comparisons of functional pharmacology have, however, not been made for orthologous arthropod 5-HT receptors except for what is reported here for 5-HT$_{1\alpha}$ and 5-HT$_{2\beta}$ from crayfish and spiny lobster. In addition, most of the studies on arthropod 5-HT receptors to date have

used binding assays or radioligand displacement assays which may not be representative of actual functional responses to the drugs. K_d measurements obtained in such assays are not directly comparable to EC_{50} values determined from dose-response curves in second messenger activation assays. The pharmacological properties of crustacean 5-HT receptors are therefore difficult to compare to their arthropod orthologs. It is, however, encouraging that we have found conservation of pharmacological properties among 5-HT receptors from relatively distantly related members of a subphylum. As more receptors from related species are characterized, we may yet find general pharmacological rules for invertebrate 5-HT receptors.

Pharmacological characterization of individual receptors in cell culture is a necessary step before applying the identified drugs to physiological preparations. In real nervous systems, however, the additional confound of non-selective drug actions becomes a concern. Because of the high conservation of key regions in 5-HT receptors, an overwhelming majority of pharmacological agents are active at more than one class of 5-HT receptors. Even after nearly 30 years of study, only a handful of truly selective drugs for human 5-HT receptors are known (Hoyer et al., 2002). In addition, there is potential for cross-reactivity with other aminergic receptors and other signaling systems. Obtaining pharmacological suites of effective agonists and antagonists for each receptor is therefore key to dissecting serotonergic signaling within intact nervous systems. However, because plasticity occurs at multiple levels even in simple invertebrate systems, pharmacological investigations should be part of an integrated approach to understanding the effects of 5-HT modulation.

Species differences in crustacean 5-HT receptor systems

Interestingly, while we have shown here that $5\text{-HT}_{2\beta}$ and $5\text{-HT}_{1\alpha}$ are well conserved at multiple levels, the 5-HT systems of *Panulirus* and *Procambarus* are

evolutionarily quite differentiated, at least in the STNS. While 5-HT functions as a synaptic neurotransmitter in clawed lobsters (*Homarus*) and crabs (*Cancer*), it is not found in the neuropil of the spiny lobster STNS but acts at very low concentrations as a neurohormone (Beltz et al., 1984). Higher concentrations of 5-HT release could occur if typically non-serotonergic neurons were able to take up, or 'borrow', free 5-HT and then release it as a transmitter. Such uptake has been observed in crayfish hindgut neurons (Musolf, unpublished) and has been suggested in the STG (Richards et al., 2003). Serotonergic neuropil is found in the STG of four crayfish genera, including *Procambarus clarkii* (Tierney et al., 1999), indicating that these systems may be configured more like those in *Homarus* and *Cancer*. Additionally, the effects of 5-HT on individual, synaptically isolated cells of the pyloric circuit vary depending on the species examined (Katz and Tazaki, 1992; Katz and Harris-Warrick, 1999). These findings indicate that the serotonergic modulatory system in this defined circuit is has differentiated through crustacean evolution. Because, as we show, the 5-HT receptors themselves are quite conserved, this differentiation must stem from expression changes or respecification of signaling properties of the conserved receptors.

Neuromodulatory mechanisms as a 'gear shift' signal for transitions between physiological states in crustaceans.

Descending neuromodulatory input maintains some aspects of physiological state in the Panulirus *STG.*

In the STG we found that two distinct states of the pyloric circuit were defined by the cycle frequency in the intact preparation and the response to 5-HT. While cycle frequency is significantly faster in intact Class I preparations, the basal cycle frequencies in the two states are indistinguishable after sucrose block of modulatory input to the STG. This indicates that some modulatory mechanism originating in anterior ganglia

may be partially responsible for defining the physiological state of the pyloric circuit. Because the *Panulirus* STG is not innervated by 5-HT fibers, this modulation must be mediated by a different transmitter. *Panulirus* STG neurons do, however, respond to low concentrations (nM) of 5-HT (Beltz et al., 1984) and the responses to 10^{-7}M 5-HT were weaker but qualitatively the same as those obtained with higher concentrations (Flamm and Harris-Warrick, 1986a). This suggests that neurohormonal 5-HT may still be involved in restructuring the 5-HT system within the pyloric circuit in an intact animal. State differences are maintained after sucrose block in the form of differential responses to 5-HT, indicating that intrinsic properties of the circuit underlie some aspects of these differences.

5-HT$_{1\alpha}$ is well placed to perform a modulatory role in response to global 5-HT signals in crayfish nerve cord.

In the nerve cord of crayfish we found that 5-HT$_{1\alpha Pro}$ might act to mediate second-order modulatory functions that could restructure circuits. 5-HT$_{1\alpha}$ immunoreactivity was widely distributed throughout the nerve cord in cell bodies and neuropil of all ganglia. In all neuropil regions we found a subset of processes that labeled for only the receptor and not 5-HT. This pattern suggests that these 5-HT$_{1\alpha}$ receptors respond to paracrine or neurohormonal signals. Most of the somata that label for 5-HT$_{1\alpha}$ are thought to be interneurons and may provide modulatory targets for 5-HT effects. In addition, 5-HT$_{1\alpha}$ was localized to processes and somata in proximal regions of the thoracic nerves and in the eyestalk; both of these areas have been implicated in neurohormonal release of crustacean hyperglycemic hormone in response to 5-HT (Sáenz et al., 1997; Chang et al., 1999; Lee et al., 2000; Lee et al., 2001). These localization patterns and the broad distribution of 5-HT$_{1\alpha}$ throughout the nerve cord indicate that, in addition to being localized to synapses, this receptor could act as a general modulator of circuits throughout

the animal. Crustaceans have an open circulatory system and frequently use paracrine and neurohormonal signaling pathways. Indeed, with an EC_{50} of 31nM, 5-HT$_{1\alpha Pro}$ is well suited to monitor resting 5-HT levels of around 5nM in the hemolymph and nerve cord of crustaceans (Sneddon et al., 2000; Panksepp et al., 2003).

5-HT may act as a 'gear shift' between physiological states while also being a substrate for state changes.

These data indicate that 5-HT receptors are optimally placed to detect neurohormonal signals that might result in state changes in crustacean nervous systems. Such neuromodulator-driven restructuring could give rise to circuits with specific properties that result in different responses to transient modulatory signals. State-dependent differences in responses to 5-HT in crustacean systems suggest that 5-HT receptors themselves may be a target in the process of reconfiguration.

How does the same 5-HT signal have state-specific effects in crustacean circuits?- changes in 5-HT receptor expression?

Within the crab STG, different cell types appear to express specific complements of 5-HT receptors (Zhang and Harris-Warrick, 1994). These cell-specific expression patterns may change as a function of the physiological state of the system in response to neuromodulatory signals.

5-HT receptors may be differentially expressed in individual identified neurons

In mammals, several proteins, including 5-HT receptors, show altered gene expression at a systemic level associated with state changes such as sleep and hibernation, stress, obesity and schizophrenia (Castensson et al., 2003; Cirelli and Tononi, 2004; Huang et al., 2004; van Breukelen et al., 2004; Dwivedi et al., 2005). In addition,

expression levels of 5-HT receptors change after exposure to agonists within specific brain regions and in cell culture (Wohlpart and Molinoff, 1998a; Riad et al., 2001). Changes in expression patterns of multiple receptors within specific neurons could therefore account for the state dependent changes observed in the STG and LG circuits (Figure 5-1A). Now that some crustacean 5-HT receptors have been cloned, single cell quantification of mRNA levels in the cells of these circuits (Baro et al., 1994; Baro et al., 1996) can be used to address this hypothesis. Until then, data from the previous chapters suggest that 5-HT receptors may be differentially expressed in a state-dependent manner.

Differential expression of 5-HT receptors in multiple neurons may generate state-specific responses in the STG

The pyloric circuit exists in two distinct physiological states defined by an increase (Class I) or reduction (Class II) in cycle frequency in response to 5-HT (Chapter 3)(Beltz et al., 1984; Flamm and Harris-Warrick, 1986a). Using drugs whose effects on $5\text{-HT}_{2\beta Pan}$ and $5\text{-HT}_{1\alpha Pan}$ were known, state-dependent modulation of the pyloric cycle by 5-HT was investigated. We found that, regardless of the state of the pyloric circuit, mCPP, which activates $5\text{-HT}_{1\alpha Pan}$ but not $5\text{-HT}_{2\beta Pan}$, always mediated a slow increase in cycle frequency in response to 5-HT. In contrast, the effect on pyloric cycle mediated by $5\text{-HT}_{2\beta Pan}$ was state-dependent. These findings suggested that expression of the $5\text{-HT}_{2\beta Pan}$ receptor could change is a state-dependent manner. $5\text{-HT}_{2\beta Pan}$ is expressed by multiple cells within the pyloric circuit (Clark et al., 2004). One of these was physiologically determined to be the AB pacemaker neuron which is known to be excited by 5-HT when synaptically isolated (Flamm and Harris-Warrick, 1986b; Ayali and Harris-Warrick, 1999) and is excited via $5\text{-HT}_{2\beta Pan}$ in Class I preparations. In Class II preparations, serotonergic reduction in pyloric cycle frequency could result from altered $5\text{-HT}_{2\beta Pan}$ expression in the AB or other pyloric neurons (Figure 5-1A). Because $5\text{-HT}_{2\beta Pan}$ appears to mediate
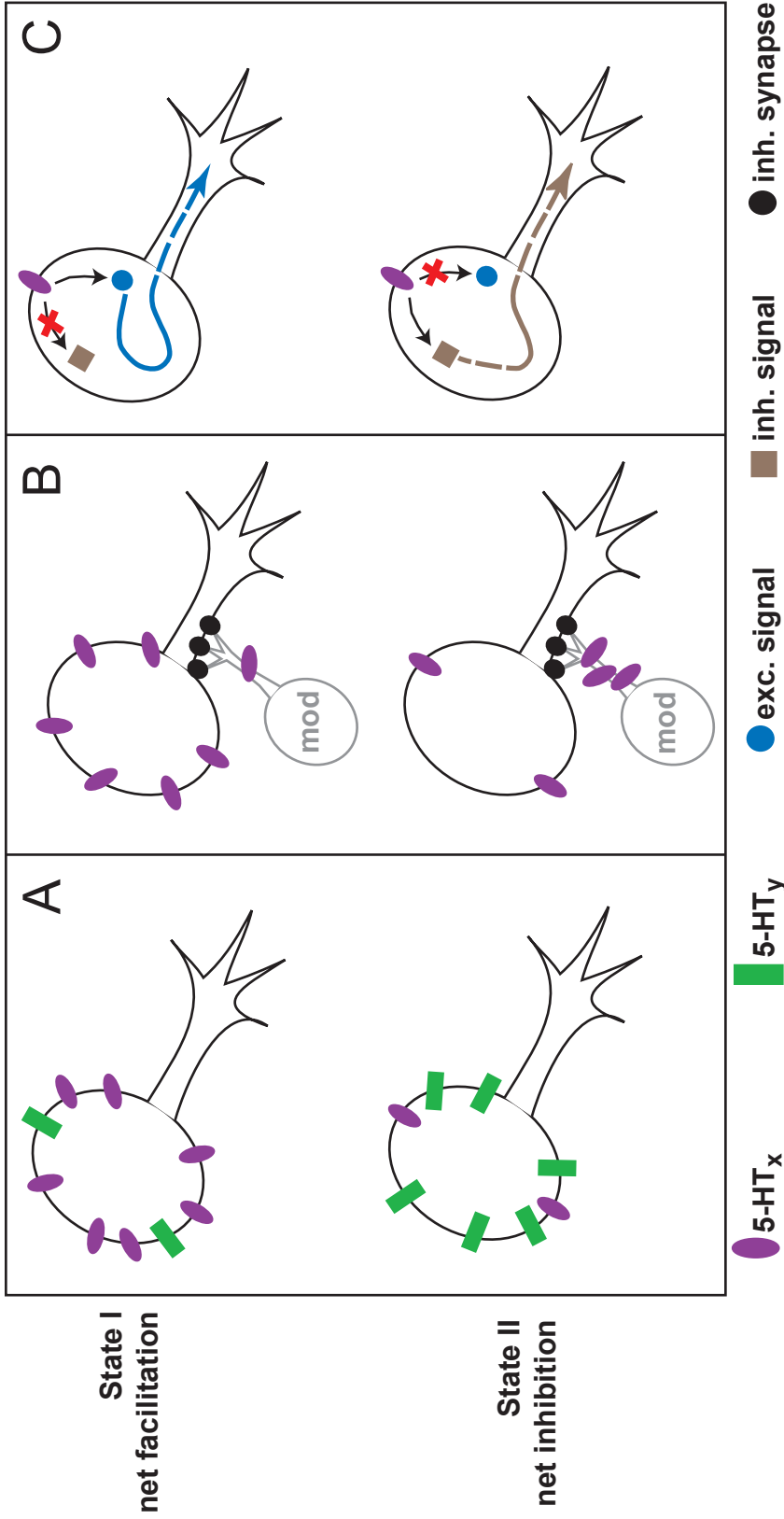
Figure 5-1: A 5-HT receptorcentric view of multiple mechnanims that could lead to state-specific 5-HT responses. **A**: The output of a neuron in the circuit determines the directionality of the 5-HT response (facilitation or inhibition). Depending on relative expression levels of excitatory (5-HT$_x$) and inhibitory (5-HT$_y$) receptors the net effect of a single neuron is changed. **B**: Relative expression of a single excitatory receptor (5-HT$_x$) is changed in the output neuron AND in a modulatory neuron (mod) that inhibits the output neuron. **C**: The same receptor population couples to different signaling cascades with opposite net effects in a state–dependent manner.

opposing effects, however, differences in expression of this single receptor in one particular cell is unlikely to account for the state differences. More likely, $5\text{-HT}_{2\beta Pan}$ and/or $5\text{-HT}_{1\alpha Pan}$ could be localized on the axon terminals of one or more modulatory neurons that interact with members of the pyloric circuit in the neuropil. Modulatory neurons could thus change their response to local 5-HT and thereby alter the directionality or magnitude of their effects on neurons of the pyloric circuit (Figure 5-1B). Such metamodulatory mechanisms have been described in several vertebrate and invertebrate systems (Katz and Edwards, 1999; Edwards et al., 2002; Mesce, 2002) including the STG where co-modulatory effects elicited by multiple transmitters released together are very important (Nusbaum et al., 2001). If 5-HT receptor expression was altered on such an indirect modulatory pathway and/or in pyloric neurons, such as AB, in preparations of different states, application of 5-HT might elicit differential effects like those observed in our experiments.

Differential $5\text{-HT}_{1\alpha}$ expression in the crayfish nerve cord.

In addition to acting as a neurohormonal receptor, $5\text{-HT}_{1\alpha}$ may change its actions in a state-dependent manner in the crayfish nerve cord. The LG tailflip escape circuit responds differentially to 5-HT depending on the concentration and speed of application suggesting the presence of multiple 5-HT receptors with differential sensitivities and signaling kinetics (Teshiba et al., 2001). Social experience changes the effects of 5-HT on the LG escape circuit such that the command neuron responds differently to sensory afferent input depending on the social status of the animal (Yeh et al., 1996; Yeh et al., 1997). In addition, the application of mCPP, which activates $5\text{-HT}_{1\alpha}$ but not $5\text{-HT}_{2\beta}$, can elicit the inhibitory component of the LG response in both dominant and subordinate animals. Because the 5-HT levels in individual ganglia of the nerve cord of crayfish do not vary with social status (Panksepp et al., 2003), it could be the 5-HT response

system that is changed in a state-dependent manner. Indeed, high variability in 5-HT$_{1\alpha}$ immunoreactivity and mRNA levels were observed amongst individual crayfish. The receptor changes associated with different social states in crayfish may therefore occur at various pathways contributing to the net modulation of the LG circuit as discussed above for the STNS of *Panulirus*.

State-dependence of modulation could also occur through reconfiguration of 5-HT receptor signaling mechanisms.

While differential expression of multiple 5-HT receptors may contribute to the different responses to 5-HT in circuits of different states, several alternate mechanisms are equally probable. For example, the signaling activation or downstream effects of a single receptor class could change in a state-dependent manner while receptor expression levels are unchanged (Figure 5-1C). Signal specificity of G protein coupled receptors (GPCRs) can depend on a multitude of factors including the localization patterns of receptor subtypes and associated machinery in microdomains within a cell, and on altered signaling pathways.

The same 5-HT receptor may alter its signaling properties in different states

The traditional view of signaling by 5-HT receptors, and GPCRs in general, is that receptors of a certain class couple preferentially to a specific G protein resulting in the release of an activated Gα subunit which goes on to initiate downstream signaling cascades. While this remains correct in general terms, it is an extreme oversimplification of what is now known to occur in cells. Not only are the Gα mediated signals subject to modulation by accessory proteins and even Gα switching but the G$\beta\gamma$ subunit is quite capable of activating signaling cascades after dissociation of the Gα subunit. In addition, a staggering array of signaling pathways that are completely independent of

G-proteins have been recently described. These pathways are now understood to be so important and prolific within systems that reversion to the original nomenclature of '7 transmembrane receptors' has been suggested for the GPC receptor family (Brzostowski and Kimmel, 2001).

Modulation of the traditional Gα mediated signaling pathways can occur via accessory proteins within the scaffold that defines a receptor's microdomain. Accessory proteins are ones that do not belong to the GPCR – G protein – classical effector triad (Sato et al., 2006) and that can alter the strength, efficiency or specificity of signal transduction at the GPCR – G protein interaction or downstream at effector activation. One class of accessory proteins that has been well characterized are the regulator of G protein signaling (RGS) proteins. RGS proteins have now been shown to bind to a variety of proteins besides Gα, including GPCRs themselves, effector molecules and other auxiliary molecules (reviewed in Abramow-Newerly et al., 2006; Sato et al., 2006). Interactions with GPCRs can directly affect signal transduction by the receptor. For example, RGS2 binds the third intracellular loop of the M1 muscarinic acetylcholine receptor to modulate G protein signalling (Bernstein et al., 2004). Alternatively, the interaction can serve a scaffolding role to stabilize the receptor within the signaling complex or microdomain at the cell surface (Hall and Lefkowitz, 2002; Bockaert et al., 2003). Differential expression of accessory proteins such as these or changes in the construction of the microdomain around the receptors could therefore be configured in a state specific manner and be responsible for distinct responses to the same neurotransmitter in different states.

Upon activation of a G protein by a receptor, the Gα subunit exchanges GDP for GTP and remains membrane-bound but dissociates from the trimeric complex to interact with downstream effectors. The remaining Gβγ complex maintains association with the membrane and can regulate several signaling cascades including adenylyl cyclases,

various kinases, ion channels and many others irrespective of the actions of Gα (Cabrera-Vera et al., 2003). Recently a role in presynaptic inhibition was also identified for Gβγ (Blackmer et al., 2005; Gerachshenko et al., 2005; Sullivan, 2005). These studies show that Gβγ binds to SNAP-25 of the synaptic vesicle fusion machinery and thereby competes with binding of SNARE, a necessary step for fusion and transmitter release. The responses resulting from the Gα and Gβγ pathways can be completely contradictory with one another within the same cell. A recently cloned and characterized arthropod dopamine receptor, for example, inhibits adenylate cyclase via the traditional $G\alpha_{i/o}$ pathway but increases cAMP concentration as a downstream effect of PLCβ activation by the Gβγ subunit (Clark and Baro, in review). Such opposing effects may act within distinct microdomains to generate specific signals regionally within a cell (Rich et al., 2001; Zaccolo and Pozzan, 2002). Alternatively, the two signaling cascades could work on different time scales and thereby contribute to either immediate or longer-lasting effects of sustained neuromodulation. An example of this type of signaling diversity has been described for the angiotensin II receptor which responds quickly with G protein mediated activation of ERK and with a slower and more sustained signal via β-arrestin cascades (Ahn et al., 2004). An interesting twist to the Gα versus Gβγ signaling multiplicity was described for the β-adrenergic receptor (Daaka et al., 1997; Lefkowitz et al., 2002). Upon activation, this receptor couples to its prototypical partner, $G\alpha_s$, resulting in a cAMP-mediated activation of protein kinase A. This enzyme proceeds to phosphorylate the receptor, switching its coupling preference to $G\alpha_i$ and terminating the $G\alpha_s$ signal. $G\alpha_i$ dissociates from Gβγ which proceeds to activate a signaling pathway terminating in the activation of ERK. Thus, activation of a single receptor has prototypical short-term effects on second-messengers such as cAMP and distinct long-term effects that may result in changed gene expression.

G protein mediated signaling is therefore not simple, can be highly unpredictable and plastic and is capable of affecting cellular responses on different spatial or temporal scales. As such, modifications in these signaling cascades could be responsible for establishing different physiological states. The contributions to the net cell response of Gα and Gβγ signaling pathways could conceivably be different in preparations of different physiological states. Such changes in the configuration of the signaling machinery may result in different responses to modulatory input at the same GPCR in a state-dependent manner. Further *in situ* studies of characterized crustacean 5-HT receptor targets in physiological systems of distinct states may determine if signaling is changed in these preparations.

In a similar manner, 5-HT receptors could have altered coupling to G protein independent signaling cascades in different states. Numerous pathways involving GPCR activation of kinases, other enzymes and transcription factors have been identified as proceeding without hetereotrimeric G proteins (Brzostowski and Kimmel, 2001; Raymond et al., 2001). GPCRs can form oligomers with distinct properties (Hansen and Schmidt, 2004) and interact directly with receptors of other molecular families to directly influence cellular responses to synaptic signals. Dopamine receptors, for example, interact directly with NMDA or AMPA receptors thereby providing a direct pathway for dopaminergic modulation of glutamate signaling (Lee and Liu, 2004; Zou et al., 2005). Modulation of these pathways in combination with those discussed above offers endless possibilities for alterations resulting in differential construction and/or activation of GPCR signaling cascades in a state-dependent manner. The signal transduction field is currently exploding and provides new tools and targets to investigate these mechanisms every week. While much of the initial characterization is done in mammalian systems and cell lines, these tools and principles can be applied to invertebrate systems where the detailed knowledge about cellular and circuit properties will enhance the power of

these studies (Clark and Baro, 2006). Now that tools such as those described in the previous chapters are being developed for crustaceans, the mechanisms underlying state changes and modulation can be investigated. Well-described invertebrate circuits such as the STNS or LG escape circuit of crustaceans will therefore prove highly valuable in understanding the basic cellular mechanisms underlying establishment, maintenance and modulation of physiological systems in distinct states.

Implications and Future

All animals proceed through multiple physiological states throughout their lives. These include natural state changes such as sleep and developmental progress and pathological states such as depression, stress and schizophrenia. Diverse physiological systems are affected and must adapt to these changes, on a wide range of timescales. Defined invertebrate circuits provide accessible systems to investigate the cellular basis of these changes in a mechanistic fashion. As discussed above, by having a 'handle' on the receptors mediating modulatory signals, we can begin to investigate the molecular infrastructure underlying state dependent changes.

Undoubtedly, several or even all of the mechanisms discussed above will be instrumental in shaping state dependent modulatory responses. But how are these states established in the first place? What signals direct the reconfiguration of the circuit? When does a 5-HT signal simply mean 'go faster for a minute' as opposed to 'restructure the signaling pathway in cell X'? Do changes occur in modules at specific circuits within the animal? If yes, how are these circuits coordinated to maintain a functional whole? Invertebrate systems will be especially suited to investigating such questions with recently developed tools because they contain distinct, accessible circuits exhibiting very stereotyped states. Because of the complex and multi-tiered nature of modulatory plasticity, no one toolset will provide a 'silver bullet' in defining the mechanisms

involved. Instead, identified circuits and cells in invertebrates allow an integrated approach that combines multiple tools to investigate the detailed synaptic, cellular and systematic mechanisms underlying plasticity at multiple levels ranging from molecular restructuring to behavior.

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