Structural and Kinetic Studies of Drug-Resistant Mutants of HIV-1 Protease

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ABSTRACT

The employment of HIV-1 protease (PR) inhibitors (PIs) in antiviral therapy has been successful in reducing mortality of HIV/AIDS patients. However, the long-term efficacy of PIs is challenged by the rapid emergence of drug-resistant mutants of PR. To understand the underlying mechanism of drug resistance, structures and activities of HIV-1 PR and its drug resistant mutants have been extensively studied. Here, PR mutants PR<sub>R8Q</sub>, PR<sub>D30N</sub>, PR<sub>I47V</sub>, PR<sub>I50V</sub>, PR<sub>I54M</sub>, PR<sub>V82A</sub>, and PR<sub>N88D/S</sub> bearing single substitutions have been investigated by crystallography and kinetics.

GRL-0519 is a potent new antiviral inhibitor of HIV-1 PR that possesses tris-tetrahydrofuran (tris-THF) as the P2 ligand. The crystal structures of GRL-0519 were determined at resolutions of 1.06-1.49 Å in complex with the mutants PR<sub>R8Q</sub>, PR<sub>D30N</sub>, PR<sub>I50V</sub>, PR<sub>I54M</sub>, and PR<sub>V82A</sub>. I50V lost its interaction with inhibitor while V82A and I54M compensated for the mutation through the main chain shift and flexibility of 80’s loop (residues 78-82), respectively. The structural changes may account for the worst inhibition of GRL-0519 for PR<sub>I50V</sub> (60-fold decrease relative to wild-type enzyme) and moderate inhibition for
PR_{I54M} and PR_{V82A} (6-7-fold decrease). The large tris-THF group at P2 provides a good fit in the S2 subsite and may be effective against resistant virus with mutations of residues in this subsite.

SQV and DRV are two clinical inhibitors that were designed to target the wild type PR and its drug resistant mutants, respectively. The crystal structures of PR mutants PR_{I47V}, PR_{N88D/s} in complex with DRV and mutants PR_{I47V} and PR_{N88D} in complex with SQV with resolutions of 1.13-1.72 Å were also analyzed. Mutation I47V gained more hydrophobic interactions with DRV and SQV. Interestingly, the structural changes did not affect the inhibition of both inhibitors for PR_{I47V} (relative $K_i$ is 0.7 and 1 for DRV and SQV, respectively). DRV and SQV showed 8-fold increase in $K_i$ for PR_{N88D} and only very subtle local changes have been observed on the structures. DRV induced 0.3 fold reduction in $K_i$ for PR_{N88S} and the distal structural changes have been transferred to the active site. This study provided fundamental information for understanding drug resistance and future design of potential antiviral drugs.

STRUCTURAL AND KINETIC STUDIES OF DRUG-RESISTANT MUTANTS
OF HIV-1 PROTEASE

by

HONGMEI ZHANG

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
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in the College of Arts and Sciences
Georgia State University
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STRUCTURAL AND KINETIC STUDIES OF DRUG-RESISTANT MUTANTS
OF HIV-1 PROTEASE

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
</tr>
<tr>
<td>PI</td>
<td>clinical inhibitor of PR</td>
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<tr>
<td>DRV</td>
<td>darunavir;</td>
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<tr>
<td>SQV</td>
<td>saquinavir</td>
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<tr>
<td>tris-THF</td>
<td>tris-tetrahydrofuran</td>
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<tr>
<td>PR /PR&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>wild type HIV-1 protease</td>
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<td>PR&lt;sub&gt;R8Q&lt;/sub&gt;</td>
<td>PR with R8Q mutation</td>
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<td>PR&lt;sub&gt;D30N&lt;/sub&gt;</td>
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<td>PR with N88S mutation</td>
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<td>RMS</td>
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1 INTRODUCTION

1.1 General Introduction of HIV/ AIDS

1.1.1 Current status of disease

Acquired immunodeficiency syndrome (AIDS) is a life-threatening disease that interferes with the immune system. There are approximately 70 million people have been infected with the HIV virus since the first case was reported in the United States in 1981. At the end of 2012, approximately 35 million people were globally living with HIV/AIDS and about 69% of them from Sub-Saharan Africa (Maghina, Govind et al. 1998, UNAIDS, 2013). Although there is no effective vaccine available (Girard and Bansal 2008), highly active antiretroviral therapy (HAART) has greatly improved the lives of AIDS patients (Brenner, Turner et al. 2002). HAART employs a combination of different antiretroviral drugs to strengthen the immune system and maximally suppress the HIV virus. The AIDS related mortality has declined in recent years due to the scaling up of effective antiretroviral therapy and the reduction of HIV incidence. It has been reported that there were 1.7 million people who died of AIDS-related illnesses in 2011, down 24% from 2.3 million in 2005. However, HIV disease is the top 6th cause of death and continues to be a major health problem in the world, especially in Africa. Therefore, intensified efforts are needed to develop more effective and low-cost drugs to combat HIV/AIDS.

1.1.2 HIV-1 life cycle

AIDS is a severe immunological disease caused by human immunodeficiency virus (HIV), a member of the retroviruses that are characterized by RNA genomes. Although HIV comprises two major subtypes HIV-1 and HIV-2, HIV-1 is the virus that was originally discovered and is more infective (Gilbert, McKeague et al. 2003). In addition, HIV-1 infections predominate globally whereas HIV-2 is largely found in West Africa (Reeves and Doms 2002). The features of HIV virus are indicated in Figure 1.1. The conical capsid of HIV virus contains two copies of the single-stranded viral RNA genome with
genes encoding for the three large polyprotein precursors Gag, Gag-Pol, and Env and several small accessory proteins Nef, Ref, Tat, Vif, Vpr and Vpu. It also has three essential viral enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN) in its capsid. The capsid of the viron particle is surrounded by a matrix, which is further surrounded by a lipid bilayer embedded by envelope proteins from the host cell. The virus enters the host cell and replicates itself through several stages (Figure 1.2). It begins infection by recognizing and binding to host cell receptor CD4 through its docking glycoprotein gp120. This process is accomplished under the help of co-receptor CCR5 and CXCR4 from the host cell. The virus then fuses with host cell membrane and enters the cell (Madden, Dalgleish et al. 1986; Deng, Liu et al. 1996; Doranz, Rucker et al. 1996). Following cell fusion and entry, the viral RNA is released to the host cell and reverse transcribed into viral DNA by the HIV RT in cytoplasm. The viral DNA is then transported across the nucleus and integrated into host cell genome by another HIV enzyme IN. Therefore, HIV viral DNA will be transcribed and translated using the host machinery. New viral RNA is produced and transported to the cytoplasm to be used as genomic RNA to synthesize polyproteins Gag, Gag-pol, Env and other accessory proteins. Gag is a polyprotein which can be cleaved to produce structural components of the virus; for example, matrix and capsid proteins. Pol contains three important viral enzymes that are essential for replication of infectious virus: RT, IN and PR. The exterior proteins SU (surface unit glycoprotein) and TM (transmembrane envelope) that recognize and bind to the host receptors are from polyprotein Env. These two proteins will be produced by cleavage of Env by cellular protease. However, Gag and Gag-Pol polyprotein precursors as well as viral RNA migrate to the cell membrane, where they assemble to form immature viral particles that are released from the infected cells. Viruses have no infectious ability until their polyproteins are cleaved into functional proteins, such as the MA, CA and NC by PR (Figure 1.3). Taken together, a mature HIV virus is produced by several steps including fusion and entry, reverse transcription, integration, translation, assembly and budding as shown in Figure 1.2 These steps could be drug targets for treatment of HIV due to their critical role in HIV life cycle.
1.1.3 **HIV-1 protease is essential for viral maturation**

HIV-1 PR is catalytically active as a homodimer. The two monomers form an active site that substrate or inhibitor can bind to and be processed. It is critical for viral particle maturation since it cleaves the viral precursor polypeptides Gag and Gag-Pol into the mature structural and enzymatic proteins (Darke, Nutt et al. 1988; Oroszlan and Luftig 1990). Inactivation of PR will interrupt the cleavage of its substrate, therefore, preventing the formation of mature and infectious virus (Kohl, Emini et al. 1988; Seelmeier, Schmidt et al. 1988). PR is regarded as an effective target for antiviral drugs due to its essential function in virus replication. The availability of PR inhibitors as clinical drugs in antiretroviral therapy dramatically improved the life quality of HIV/AIDS patients (Brenner, Turner et al. 2002).

The compositions of the Gag and Gag-Pol polyproteins are shown in Figure 1.3. Cleavage of Gag by PR produces structural proteins MA, CA, p2, NC, p1 and p6 (Henderson, Bowers et al. 1992) (Wondrak, Louis et al. 1993). In addition to the first five structural proteins from Gag, Gag-Pol also produces several viral enzymes, including RT, IN and PR (Louis, Weber et al. 2000). PR itself is released from the precursor Gag-Pol as an active dimer by autoprocessing (Louis, Nashed et al. 1994; Louis, Wondrak et al. 1999). Both *in vitro* and *in vivo* studies have shown that PR cleaves the Gag-Pol precursor at specific sites and in a specific order (Pettit, Moody et al. 1994; Wiegers, Rutter et al. 1998; Pettit, Henderson et al. 2002). Figure 1.3 lists the cleavage sites in Gag and Gag-Pol and the sequential order of cleavage. The p2/NC site is cleaved first, followed by MA/CA. Cleavage of the sites on either side of PR was the last two steps. It has been reported that correct order of processing is also essential for proper assembly and viral maturation (Oroszlan and Luftig 1990; Rose, Craik et al. 1998).

1.2 **The structural properties of HIV-1 PR**

Great efforts have been made to determine the three dimensional structures of PR in order to serve as guides for design of antiviral protease inhibitors (PIs). The first X-ray crystal structures of PR were determined with resolution of approximately 3 Å in 1989 (Lapatto, Blundell et al. 1989; Miller, Schneider et al. 1989; Navia, Fitzgerald et al. 1989; Wlodawer, Miller et al. 1989). Higher resolution
structures have been obtained with the technical advances in X-ray detectors and availability of high intensity synchrotron sources for X-rays (Tie, Boross et al. 2004; Liu, Boross et al. 2005; Tie, Kovalevsky et al. 2007). In 2006, the X-ray crystal structure of PR mutant bearing single substitutions of V32I was solved at ultra-high resolution of 0.84 Å (Kovalevsky, Liu et al. 2006). Up to date, there are more than 400 HIV-1 PR structures unliganded or complexed with various inhibitors or substrates have been deposited in the Protein Data Bank (Berman, Westbrook et al. 2000).

HIV-1 PR is enzymatically active as a dimer of two 99-residue subunits. It belongs to the aspartic protease family which is characterized by the conserved catalytic triplet sequence of Asp-Thr/Ser-Gly (Darke, Nutt et al. 1988). The structure of PR is predominantly composed of β-sheet with a very short α-helix near the C terminus (residues 86–94) as shown in Figure 1.4 (A). Inhibitors or substrates bind the active site between two subunits through hydrogen bonds and van der Waals interactions. Two aspartic acids at residue 25 from two subunits form the central active site and are essential for enzyme activity. The mutation of Asp25 leads to complete loss of the enzyme’s ability to hydrolyze the substrate peptide bonds (Kohl, Emini et al. 1988; Seelmeier, Schmidt et al. 1988).

Another important region in the PR structure is the flexible flaps. HIV-1 PR possesses two flap regions, one from each subunit comprising residues 45 to 56. The flaps are very flexible and were observed in different conformations depending on whether the substrate or inhibitor is bound. This flexibility is very important to the catalytic activity of PR. The closed conformation ensures the tight binding of the substrate or inhibitor. The closed conformation with the inhibitor also blocks the processing of its substrate once the competitive PI is bound to the catalytic site (Darke, Nutt et al. 1988; Mahalingam, Louis et al. 1999; Scott and Schiffer 2000; Weber and Agniswamy 2009). The state of the flap can also be affected by the mutation in the flap regions, thus mutation of the flap residues has great impact on the PR activity and substrate or inhibitor binding affinity as shown in Figure 1.4B (Liu, Kovalevsky et al. 2006). It has been shown that the Gly-rich region (Gly48, Gly49, Gly51, Gly52) in the flap is more sensitive to substitution (Shao, Everitt et al. 1997). However, flap residues (Ile47, Ile50, Ile54 and Val56) whose side chains are directed inward and may interact directly or indirectly with the substrate and inhibitor could
tolerate a few mutations (Shao, Everitt et al. 1997). Molecular dynamic simulations and NMR experiments also demonstrated that the flap is in the dynamic equilibrium of open, partially open and closed conformation (Tozser, Blaha et al. 1991; Coffin, Hughes et al. 1997; Bagossi, Sperka et al. 2005). Indeed, our most recent study of a clinically derived extreme HIV-1 PR mutant (PR20) bearing 20 mutations revealed three distinct conformations of the flexible flaps from structures of free PR20, peptide bond and inhibitor bound PR20 (Agniswamy, Shen et al. 2012). The remarkable variety of flap conformations may influence the catalytic activity and stability of PR and contribute to resistance mechanisms (Liu, Kovalevsky et al. 2006; Agniswamy, Shen et al. 2012).

The catalytic Asp25 from each subunit and the flap regions play important roles in enzyme activity and stability. In addition, the ionic interactions of Arg 8 with Asp 29' and Arg 87', the N-terminus (residues 1-4) and C-terminus (residues 96-99) form the dimer interface and also influence the dimerization significantly (Louis, Ishima et al. 2003). Substitution of Arg by Gln at residue 8 resulted in the loss of ionic interaction with Asp29 and thus decreased stability and catalytic activity (Weber 1990; Mahalingam, Louis et al. 1999). Finally, Gly86-Arg87-Asn88 is the second conserved triad in retroviral proteases (Pearl and Taylor 1987; Louis, Weber et al. 2000). Although residues Gly86-Arg87-Asn88 that are located in the α-helix do not directly interact with the substrate or inhibitor, they are close (with indirect interactions) to the active site loop and may contribute to dimer formation (Schechter and Berger 1967; Louis, Smith et al. 1989; Ishima, Gong et al. 2010).

1.3 The catalytic mechanisms of HIV-1 PR

In order to facilitate the design of novel antiviral inhibitors, intensive efforts have been put into study of the catalytic mechanism of HIV-1 PR. In fact, a general acid-base mechanism has been proposed for aspartic PR by kinetic study of pepsin-like aspartic PR (Suguna, Bott et al. 1987; Ji and Loeb 1992). In 1991, Hyland proposed the reaction mechanism specifically for HIV-1 PR based on the 18O exchange mass spectrometry experiments and structure data (Hyland, Tomaszek et al. 1991; Brik and Wong 2003; Yu, Wild et al. 2005). Similar to the general acid-base model proposed by Suguna (Suguna, Bott et al.
The catalytic Asp25 residue from subunit is un-protonated and acts as base, whereas the other Asp25 is protonated and acts as acid. An H$_2$O attacks and transfers a proton to the basic Asp25 while the acidic Asp25 attacks and protonates the substrate (Figure 1.5). There is a reversible tetrahedral intermediate formed before the peptide is hydrolyzed.

An H$_2$O molecule is involved in catalytic mechanism proposed for both pepsin-like protease and HIV-1 PR. However, the quaternary structure of HIV-1 PR is different from that of pepsin-like protease. HIV-1PR is enzymatically active as a dimer and the two catalytic Asp25 are from two subunits while pepsin-like PR is active as a monomer and the two catalytic Asp25 involved in the reaction are from two domains of the monomer. Therefore, the H$_2$O in the active site of the pepsin-like protease may not be present for HIV-1 PR since there is no space for such an H$_2$O in the active site as shown in crystal structures of HIV-1 PR with inhibitors. Another possibility could be the conserved water that is consistently observed between the NH groups of the main chain of flap residues Ile50 / Ile50’ and two C=O groups of the substrate/inhibitors (Coffin 1995). However, this conserved H$_2$O molecule is not close to the active catalytic Asp25 and has less chance to attack the scissile bond that is located on the opposite side of substrate to the way proposed in the model. The later study by Baca showed that this water does not directly interact with the catalytic Asps and substrate, which is further supported by the molecular dynamics simulation studies performed by the Chatfield group (Baca and Kent 1993; Chatfield and Brooks 1995). In 1994, Harrison proposed another model to explain how this H$_2$O molecule possibly contributes the catalytic reaction by the molecular dynamic simulations of HIV PR (Harrison and Weber 1994). In his model, the H$_2$O molecule attacks scissile bond from opposite side to form the transition-state intermediate.

In addition to the enzyme kinetics and theoretical models, X-ray crystallographic analysis is also a powerful tool to illustrate different states in catalytic mechanisms (Kuwata, Miyazaki et al. 1997). The tetrahedral reaction intermediates proposed in the model have been observed in several structures as illustrated in Figure 1.5 (Shafer 2002; Wu, Schiffer et al. 2003; Torbeev, Mandal et al. 2008). In our most recent crystallographic study, three consecutive stages in the proteolytic reaction were captured in near-atomic-resolution in HIV-1 PR and its mutants (Shen, Tie et al. 2012).
Recently, neutron crystallography, which is able to provide the critical information about the location of hydrogen atoms, has been exploited in the study of reaction intermediate. Single protonated Asp25 was observed in the structure of PR with the inhibitor KNI-272 (Adachi, Ohhara et al. 2009). The most recent joint x-ray/neutron crystallographic study of HIV-1 PR with amprenavir suggested different location of hydrogen atoms on the protonated Asp (Weber, Waltman et al. 2013). Taken together, different catalytic mechanisms have been proposed for HIV-1 PR and involvements of the nucleophilic water and the function of the two aspartic residues are commonly accepted; however, the debate continues on the detailed reaction mechanism. Neutron crystallography may shed light on reveal of the catalytic mechanisms of HIV-1 PR.

1.4 The substrate specificity of HIV-1 PR

HIV-1 PR exhibits very high specificity for the viral precursor Gag and Gag-Pol polyproteins and other peptides with the natural cleavage site sequences (Darke, Nutt et al. 1988). The substrate has a minimum of seven residues (P4-P3’) for optimal catalysis (Darke, Nutt et al. 1988; Tozser, Blaha et al. 1991; Tomasselli and Heinrikson 1994; Coffin, Hughes et al. 1997). HIV-1 PR cleaves the substrate at the scissile bond, which has four residues located on its N terminus defined as P1, P2, P3 and P4 and three residues on its C terminus defined as P1’, P2’ and P3’ as shown in Figure1.6 (Schechter and Berger 1967; Shen, Tie et al. 2012). The corresponding binding sites on PR that accommodate P4-P3’ residues are defined as subsites S4 to S3’. Residues that are involved in binding the substrate in the subsites are also illustrated in Figure1.6. The specificity has been investigated by incorporating different residues at each position (Beck, Morris et al. 2002). These studies showed that the size and chemical property of the side chain on the substrate affects the binding specificity with the PR. There are preferences for P1/P1’ and P2/P2’. P1 and P1’ favor residues with large hydrophobic and aromatic side chains whereas P2 and P2’ can be hydrophobic and small polar residues (Bagossi, Sperka et al. 2005). Understanding the polyprotein cleavage sites and the substrate specificity provides very valuable information that can be incorporated in the design of antiviral inhibitors.
1.5 Structure guided design of PIs

1.5.1 The first generation of antiviral PIs

Over the past decades, extraordinary efforts have been made in order to develop effective antiviral therapy and overcome HIV drug resistance. Up to date, there are about 25 antiretroviral drugs approved by the FDA (Food and Drugs Administration) and used to treat HIV/AIDS patients. Currently, nine of the drugs in HAART are designed to target HIV PR, including amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, atazanavir, tipranavir and darunavir (Figure 1.7). HAART therapy that uses a cocktail of different drugs has greatly improved the life quality of HIV/AIDS patients, especially after the introduction of the first PI, saquinavir, in the market (Wlodawer and Vondrasek 1998).

The information for the design of antiviral PIs was deduced from the first X-ray crystal structures of wild type HIV-1 PR complexed with non-hydrolysable peptide analogs (Miller, Schneider et al. 1989). Hydrogen bonds and hydrophobic interactions were observed between the PR and the peptide analogs. It has been shown that the main chain atoms of the peptide analogs interact with active site residues 25-29 on one side and flap residues 48-50 on the other side by conserved hydrogen bonds (Gustchina and Weber 1990; Gustchina, Sansom et al. 1994). These observations have been confirmed in the more recent high resolution structures of HIV-1 PR with substrate analogs as shown in Figure 1.8 (Tie, Boross et al. 2005). Most of the residues in the active site can form hydrophobic interactions with the peptidic inhibitor. There are several obstacles to overcome for the peptide inhibitors, however. For example, these inhibitors are usually very large and could not easily penetrate into the cells. In addition, some peptide-like compounds are very unstable and could be digested by the cellular enzymes. Therefore, the design of new inhibitors that are relatively smaller and less peptidic is necessary. The first generation of clinical inhibitors, such as saquinavir (SQV, Figure 1.7&1.9A) was designed primarily based on the crystal structures of HIV-1 PR with peptidic inhibitors. They were designed to bind tightly in the active site cavity of the wild type PR by mimicking the interactions between HIV-1 PR and peptidic inhibitors. The first generation of PIs contains polar groups that resemble the peptide-like amides and carbonyl groups, and therefore maintains its
hydrogen bonds with the main chain of the PR. It also possesses large hydrophobic groups that mimic the peptide side chains and bind in hydrophobic subsites (Figure 1.6). It has been proved that the use of these early PIs in combination with reverse transcriptase inhibitors successfully suppressed viral maturations.

1.5.2 **Structure guided design of PIs targeting drug resistant HIV-1 PR.**

Even though HAART therapy with incorporation of PIs has made tremendous progress in decreasing the mortality for HIV/AIDS patients; there are several obstacles for PIs in its long term effectiveness, including the emergence of drug resistant viral strains (Wensing, van Maarseveen et al. 2010). The presence of these mutations on PR could readily reduce its binding affinity with the earlier PIs. For drug resistance to occur, these mutations should maintain viral fitness while diminishing drug binding. Comparison of the X-ray structures of HIV-1 PR and its variants demonstrated that the main chain atoms of residues cannot easily be altered by mutation. In addition, some residues are conserved since their mutations may result in the loss of PR catalytic activity. Therefore, the strategy for design of the next generation of inhibitor is to maximize inhibitor interactions with PR backbone atoms and conserved residues that are required to maintain the critical PR function in viral replication (Ghosh, Chapsal et al. 2008). The early structure-based efforts also include reducing the peptide-like features of PIs in order to improve bioavailability.

According to the backbone-binding strategies, the active site residues become an ideal target region for inhibitor binding, thus maximizing the hydrogen-bonding interactions between the inhibitor and the protein backbone atoms in the S2–S2’ subsites becomes the focus of drug design (Ghosh, Anderson et al. 2012). Pursuit of this strategy led to the discovery of a variety of potent antiviral inhibitors including darunavir (DRV, Figure1.7&1.9B), which has been proved to be a successful for AIDS salvage therapy (Koh, Nakata et al. 2003; King, Prabu-Jeyabalan et al. 2004; Tie, Boross et al. 2004). DRV exhibits stronger binding affinity (5pM affinity) partly due to the many hydrogen bond interactions with PR (King, Prabu-Jeyabalan et al. 2004). It not only blocks the processing of the substrate but also inhibits the dimer-
ization of PR (Koh, Matsumi et al. 2007), thus acting as dual inhibitor and demonstrated high potency and clinical efficacy on resistant viral infections (Tozser, Blaha et al. 1991; Ghosh, Kincaid et al. 1998; Tie, Boross et al. 2004; Surleraux, Tahri et al. 2005). It incorporates bis-tetrahydrofuran (THF) in the P2 group, thus introducing more hydrogen bond interactions with PR main chain atoms and conserved residues (Ghosh, Xu et al. 2010). Comparison of the crystal structures of DRV with PR and resistant PR mutants have shown that the majority of the strong hydrogen bond interactions observed between DRV and PR were maintained in its interaction with drug resistant variants (Tie, Boross et al. 2004; Kovalevsky, Liu et al. 2006; Kozisek, Bray et al. 2007; Liu, Kovalevsky et al. 2008). The X-ray structures suggested that DRV may have a second binding site on the flap region of PR (Kovalevsky, Tie et al. 2006). This is further confirmed by its kinetic study, which suggested a “mixed-type competitive–uncompetitive inhibition model” (Kovalevsky, Ghosh et al. 2008). In summary, design of DRV was a milestone in the combating AIDS disease and HIV resistance.

1.5.3 Development of the next generation of PIs

Based on the backbone scaffold of DRV, a wild range of new inhibitors have been developed. These new inhibitors employed a variety of chemical groups at P2, P1’ and P2’. The chemical structures of some new inhibitors that have been developed by the Ghosh group in recent several years are illustrated in Figure 1.10. Most of them showed picomolar inhibition of PR and nanomolar range of antiviral IC\textsubscript{50} values. In addition, they exhibit high potency on cells infected by resistant virus. Inhibitors including GRL-0519A, GRL-04410, GRL-0249 and GRL-0489 have P2 groups different from DRV (Ghosh, Leshchenko-Yashchuk et al. 2009; Ghosh, Xu et al. 2010; Ghosh, Chapsal et al. 2012). X-ray crystallography study showed that the newly incorporated THF ring in GRL-0519A had enhanced interaction with conserved residues and filled the hydrophobic S2 subsite. This is consistent with the tenfold increase in its antiviral activity against multidrug-resistant strains relative to DRV (Ghosh, Xu et al. 2010). GRL-0249 is another antiviral inhibitor with modified P2 group. Structural analysis showed that it formed new hydrogen bond interactions with Gly48 in flap of PR (Ghosh, Chapsal et al. 2012). Antiviral inhibitor GRL-
02031 was developed by varying the P1’ group of DRV. It has been reported that GRL-02031 exhibited full potency against a variety of multidrug-resistant HIV-1 strains (Ghosh, Leshchenko-Yashchuk et al. 2009). In summary, these newly developed antiviral PIs with various P2 and P1’ groups showed enhanced interaction with the conserved residues and proved to be potent antiviral inhibitors. However, design of next generation of inhibitor with an improved strategy is still necessary due to the persistent emergence of drug resistance.

1.6 Molecular mechanisms of drug resistance of HIV-1 PR

1.6.1 HIV-1 PR mutations

HIV-1 PR is critical for viral particle maturation since it cleaves the viral precursor polypeptides Gag and Gag-Pol into the mature structural and enzymatic proteins (Darke, Nutt et al. 1988; Oroszlan and Luftig 1990). Thus PR is an effective target for antiviral drugs, however, the most severe challenge to the long-term efficacy of PIs in HAART is the emergence of drug-resistant mutants of PR (Richman, Morton et al. 2004). In fact, HIV-1 PR is naturally polymorphic and the polymorphisms exist in approximately half of the residues (Velazquez-Campoy, Todd et al. 2001). Regardless of the natural polymorphisms, the development of mutations is very rapid and it results in very severe problems in AIDS treatment. It has been reported that drug resistant viruses are harbored by over 70% of HIV-1 infected individuals and approximately 5-10% of them are resistant to all of the current PR inhibitors used in the clinic (Yu, Wild et al. 2005). The major causes leading to the rapid emergence of mutations are the lack of 3’-5’ exonuclease proofreading function of HIV-1 RT and rapid replication of virus particles (Ji and Loeb 1992). In an HIV-1 infected patient, there are over 109 new cells infected and 10 billion new virus particles produced every day if no treatment is received (Coffin 1995; Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995). In addition, simultaneous infection by more than one virus with different sequences may increase the genetic diversity through recombination (Robertson, Sharp et al. 1995; Kuwata, Miyazaki et al. 1997). The presence of resistant virus could also come from the drug selection during antiviral therapy. The drug resistant mutants become dominant when the wild type virus has been killed in the presence of antiviral drugs.
(Jacobsen, Yasargil et al. 1995; Wei, Ghosh et al. 1995). The drug resistant mutations on PR also affect their abilities to process the substrates, thus additional mutations emerge in order to restore their catalytic efficiency (Condra, Schleif et al. 1995). For the drug resistant HIV strains, mutations may occur on both the substrate $Gag$ and $Gag$-$Pol$ and PR in order to maintain the enzyme’s ability to normally process its substrate in the presence of the drug (Mammano, Petit et al. 1998; Tamiya, Mardy et al. 2004). The rapid emergence of the resistant mutants becomes the key challenge of antiviral therapy because these mutants still retain their protease function while reducing or eliminating susceptibility to the PIs so HIV can replicate during drug therapy (Miller 2001).

1.6.2 Molecular mechanisms of drug resistance

Mutations in up to 36 of 99 residues in HIV-1 PR are related to drug resistance for the current PIs (Wu, Schiffer et al. 2003; Johnson, Calvez et al. 2013). The positions of these mutations are mapped on Figure 1.11. These mutations are classified as major or minor mutations in terms of their effects on antiviral therapy. Major mutations usually exhibit higher levels of resistance and are able to cause drug resistance to one or more PIs themselves. On the other hand, the minor mutations show relatively lower resistance to the PIs and are likely to appear with other mutations (Shafer 2002). There are 15 major mutations and 21 minor mutations as shown in Figure 1.11. In fact, the same mutation may have different levels of resistance to different drugs. In this case, it will be regarded as major mutation on Figure 1.11. The mutation could also be classified into active site, flap region, dimer interface and distal region mutations based on their locations on HIV-1 PR.

The structures and activities of HIV-1 PR mutants have been extensively studied in order to reveal the molecular mechanisms of drug resistance. Several different mechanisms have been discovered by comparison of wild type PR and its single mutants (Weber and Agniswamy 2009). Generally, the mutations located in the inhibitor binding site lead to the decreased interaction with the drugs. These include mutations of residues Leu23, Asp30, Val32, Met46, Ile47, Gly48, Ile50, Val82, and Ile84 that interact with substrate or inhibitor as indicated in Figure 1.6. The mutations in the dimer interface primarily influ-
ence the protein stability. The classification of mutations based on the location is not absolute. For example, some mutations on the flap region residue not only affect direct interaction with the inhibitor, but also alter the interaction between two subunits and thus contribute to the reduction of PR dimer stability. This has been clearly illustrated in mutation I50V. The loss of the interaction between two subunits and the lower PR stability are also observed in other mutations such as L24I and F53L (Darke, Nutt et al. 1988; Liu, Kovalevsky et al. 2006). Thus, the reduced PR dimer stability is regarded as a widely used mechanism to produce resistance to PIs. A distinctive mechanism has been observed in active site mutation V82A, which exhibits a main chain shift to accommodate the inhibitor. However, the main chain shift will not always compensate for the loss of interaction with inhibitor caused by mutation (Tie, Boross et al. 2004; Tie, Boross et al. 2005; Tie, Kovalevsky et al. 2007). Different from the mutations stated above, the mutations located in the distal region usually have subtle and variable effects (Mahalingam, Louis et al. 2001; Mahalingam, Wang et al. 2004; Kozisek, Bray et al. 2007). The structural changes caused by these mutations, such as L90M and G73S, are transmitted to the active site, thus having an indirect impact on the interaction with inhibitors (Darke, Nutt et al. 1988; Tozser, Blaha et al. 1991; Mahalingam, Louis et al. 1999; Liu, Kovalevsky et al. 2006). Even though some mechanisms of drug resistance have been identified, drug resistance in clinical isolates may result from the combination of different subtle changes and can be much more complicated. However, the discovered underlying molecular mechanisms for drug resistance can always be incorporated into the strategies of structure guided design of antiviral PIs targeting drug resistant HIV strains.

1.7 Rationale for this study

The long-term effectiveness of antiviral therapy is severely challenged by the drug resistance and its underlying molecular mechanisms are still elusive. Up to date, hundreds of crystal structures have been determined in order to guide the design of more effective PIs to combat drug resistance. The antiviral PIs SQV and DRV were designed to target the wild type PR and its drug resistant mutants, respectively. Selective drug resistance of I47V was found to most FDA approved drugs including DRV, with SQV being
the only exception. Therefore, it would be interesting to investigate how I47V mutation exhibits different effects on DRV and SQV. The effects of a distal region mutation N88D/S on these two PIs were also studied.

The bis-THF of DRV introduces more hydrogen bonds with PR main chain atoms (Ghosh, Kincaid et al. 1998; Surleraux, Tahri et al. 2005). Recently, a third THF ring was added to enlarge P2 and fit better in the S2 binding pocket of PR, leading to the novel PI called GRL-0519. The incorporation of the third ring endows GRL-0519 with excellent antiviral activity on drug resistant virus (Ghosh, Xu et al. 2010). In order to study the molecular basis for the potency of GRL-0519 against drug resistant viral strains, the structural and kinetic effects of mutations from different regions including active site (PRD30N, PRI50V, PRV82A), flap region (PRI50V, PRI54M) and dimer interface (PRR8Q, PRI50V) were analyzed with GRL-0519. These mutations, with the exception of R8Q, are common in drug resistant clinical isolates (Johnson, Calvez et al. 2013). These studies will help improve our knowledge of the molecular basis of drug resistance and guide the design of more potent anti-viral inhibitors.
Figure 1.1 The schematic picture of the HIV virus particle shows its major components. From the website: http://www.eenzyme.com/hivresearchtools.aspx.
Figure 1.2 The life cycle of HIV.

From the website: http://cnx.org/content/m44599/latest/?collection=col11448/latest.
Figure 1.3 Gag and Gag-Pol polyproteins and HIV-1 PR cleavage sites.
HIV-1 PR cleavage sites are indicated by orange arrows. The number represents the order of cleavage of the polyproteins by HIV-1 PR with 1 for the first and 5 for the last (Wiegers, Rutter et al. 1998).
Figure 1.4 The overall structure of HIV-1 PR/inhibitor (DRV) complex.
(A) The active site Asp25 is indicated by red sticks. The flap regions and the termini (N and C terminus) at the dimer interface are represented in magenta and cyan, respectively. The inhibitor (here DRV) is shown in green lines. (B) Different conformations of flaps are showed in superposition of unliganded protease (PR$_{WT}$ in green, PDB ID: 1HHP) (Spinelli, Liu et al. 1991), unliganded protease with F$_{53L}$ mutation (PR$_{F53L}$ in red, PDB ID: 2G69) (Liu, Kovalevsky et al. 2006) and protease complex with darunavir (blue, PDB ID: 2IEN, inhibitor is removed for clarity) (Tie, Boross et al. 2004).
Figure 1.5 The scheme for a catalytic mechanism of HIV-1 PR.
Figure 1.6 Diagram of a substrate (P4-P3') with its binding sites (S4-S3') on HIV-1 PR. The scissile peptide bond is indicated by a red star. PR residues contributing to the binding site are labeled (Shen, Tie et al. 2012).
Figure 1.7 Currently approved FDA drugs for HIV-1 PR with date of approval.
Figure 1.8 Hydrogen bond interactions between PR and substrate analogs p2/NC.

Hydrogen bond interactions between PR and inhibitor in high resolution structure of HIV-1 PR with substrate analogs p2/NC at 1.4Å. Carbon atoms are colored gray for PR and yellow for peptide inhibitor. Water molecules are indicated by red spheres. The conserved and nonconserved hydrogen bond interactions between the PR and inhibitor are shown as green and black dashed lines, respectively (Tie, Boross et al. 2005).
Figure 1.9 Hydrogen bond interactions between PR and inhibitors.
Carbon atoms are colored gray for PR and cyan for inhibitor. Water molecules are indicated by red spheres. The hydrogen bond interactions between the PR and inhibitor are shown as black dotted lines. (A) Hydrogen bond interactions between PR and Saquinavir. (B) Hydrogen bond interactions between PR and Darunavir (Koh, Nakata et al. 2003; Tie, Boross et al. 2004; Tie, Kovalevsky et al. 2007).
Figure 1.10 The chemical structures of new antiviral inhibitors.

Figure 1.11 Mutations in the PR gene associated with resistance to PIs.
Major mutations and minor mutations are indicated by red and blue sphere, respectively. The positions on the PR are also labeled (Johnson, Calvez et al. 2013).
2 NOVEL P2 TRIS-TETRAHYDROFURAN GROUP IN ANTIVIRAL COMPOUND 1 (GRL-0519) FILLS THE S2 BINDING POCKET OF SELECTED MUTANTS OF HIV-1 PROTEASE

2.1 Abstract

GRL-0519 (1) is a potent antiviral inhibitor of HIV-1 protease (PR) possessing tris-tetrahydrofuran (tris-THF) at P2. The high resolution X-ray crystal structures of inhibitor 1 in complexes with single substitution mutants PR_{R8Q}, PR_{D30N}, PR_{I50V}, PR_{I54M}, and PR_{V82A} were analyzed in relation to kinetic data. The smaller valine side chain in PR_{I50V} eliminated hydrophobic interactions with inhibitor and the other subunit consistent with 60-fold worse inhibition. Asn30 in PR_{D30N} showed altered interactions with neighboring residues and 18-fold worse inhibition. Mutations V82A and I54M showed compensating structural changes consistent with 6–7-fold lower inhibition. Gln8 in PR_{R8Q} replaced the ionic interactions of wild type Arg8 with hydrogen bond interactions without changing the inhibition significantly. The carbonyl oxygen of Gly48 showed two alternative conformations in all structures likely due to the snug fit of the large tris-THF group in the S2 subsite in agreement with high antiviral efficacy of 1 on resistant virus.

2.2 Introduction

HIV/AIDS is a life-threatening disease that interferes with the immune system, and approximately 1.8 million people died of AIDS-related illnesses in 2010 (Maghina, Govind et al. 1998). Although there is no effective vaccine available (Girard and Bansal 2008), highly active antiretroviral therapy (HAART), which employs a combination of different antiretroviral drugs, improves the lives of AIDS patients (Brenner, Turner et al. 2002). HIV-1 protease (PR) is critical for viral particle maturation since it

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cleaves the viral precursor polypeptides Gag and Gag-Pol into the mature structural and enzymatic proteins (Darke, Nutt et al. 1988; Oroszlan and Luftig 1990). Thus PR is an effective target for antiviral drugs, however, the most severe challenge to the long-term efficacy of protease inhibitors (PIs) in HAART is the emergence of drug-resistant mutants of PR (Richman, Morton et al. 2004).

HIV-1 PR is catalytically active as a homodimer. Structural regions critical for PR activity and stability are the dimer interface including the catalytic Asp25 from each subunit and the flexible flaps comprising residues 45 to 55 (Mahalingam, Louis et al. 1999; Weber and Agniswamy 2009). To date, there are nine approved clinical PIs. The first clinical inhibitors, such as saquinavir (SQV), were designed to bind tightly in the active site cavity of the wild type enzyme; however, their binding affinity can be readily lowered by mutations. Analysis of the structural and biochemical properties of PR mutants suggests that resistant mutations act by multiple mechanisms, including mutations in the binding site that directly lower inhibitor affinity, mutations at the dimer interface that destabilize the catalytically active dimer, and flap mutations that alter the conformational flexibility (Weber and Agniswamy 2009). Drug-resistant PR mutants exhibit decreased binding affinity for inhibitors while maintaining the critical PR function in viral replication (Miller 2001). Two clinical PIs, darunavir (DRV) and amprenavir (APV), contain tetrahydrofuran (THF) in the P2 group; APV has a single THF while DRV incorporates bis-THF.

The bis-THF of DRV introduces more hydrogen bonds with PR main chain atoms, and DRV has demonstrated high potency and clinical efficacy on resistant viral infections (Ghosh, Kincaid et al. 1998; Surleraux, Tahri et al. 2005). Recently, a third THF ring was added to enlarge P2 and fit better in the S2 binding pocket of PR, leading to the novel PI called GRL-0519 (1) (Figure 1A). The incorporation of the third ring endows inhibitor 1 with excellent antiviral activity on drug-resistant virus (Ghosh, Xu et al. 2010).

The crystal structure of inhibitor 1 complexed with wild type PR (PR\textsubscript{WT}) was reported previously (Ghosh, Xu et al. 2010). The third THF ring showed new water-mediated hydrogen bonds with conserved PR residues Gly27, Asp29 and Arg8’. In order to study the molecular basis for the potency of inhibitor 1 against drug-resistant viral strains, crystal structures of inhibitor 1 complexes with PR mutants bearing
single substitutions of R8Q, D30N, I50V, I54M and V82A (PR_{R8Q}, PR_{D30N}, PR_{I50V}, PR_{I54M} and PR_{V82A}) were analyzed. The location of these mutations in the PR dimer is indicated in Figure 1B. These mutations, with the exception of R8Q, are common in drug resistant clinical isolates (Johnson, Calvez et al. 2011). R8Q was one of the first resistant mutants identified in the laboratory for an investigational inhibitor (Ho, Toyoshima et al. 1994). In the wild type enzyme, Arg8 forms an ionic interaction with Asp29’ in the other subunit as an important component of the dimer interface (Ishima, Gong et al.; Weber 1990). This intersubunit ionic interaction was eliminated in the mutant with the single substitution of R8Q (Mahalingam, Louis et al. 1999). Moreover, in the PR_{WT}-inhibitor 1 complex, the side chain of Arg8 forms a water-mediated hydrogen bond with the third THF of inhibitor 1 (Ghosh, Xu et al. 2010). Therefore, it is of particular interest to test how the R8Q mutation affects the binding of inhibitor 1. D30N is a major mutation that is associated with resistance to nelfinavir (NFV) (Jarvis and Faulds 1998). Asp30 forms hydrogen bond interactions with the bis-THF of DRV, thus mutation of this residue may alter the inhibitor binding. Mutations of the flap residues such as Ile50 and Ile54 can alter the conformational dynamics of this region, thereby affecting the binding affinity for inhibitors (Pazhanisamy, Stuver et al. 1996; Liu, Boross et al. 2005; Liu, Kovalevsky et al. 2006; Liu, Kovalevsky et al. 2008). Ile50 is located at the tip of the flap where its side chain forms hydrophobic interactions with inhibitors. Mutation of I50V to a shorter side chain is expected to reduce the binding affinity for inhibitors. Indeed, PR with I50V mutation exhibits significantly reduced inhibition by indinavir, SQV, and DRV (Liu, Boross et al. 2005; Kovalevsky, Tie et al. 2006; Liu, Kovalevsky et al. 2008). I50V also has a significant effect in destabilizing the PR dimer (Liu, Boross et al. 2005). Mutations of Val82 are found frequently in resistant virus (Johnson, Calvez et al. 2011). The mutation V82A in the active site cavity can eliminate interactions with inhibitor, and also exhibits a shift of its main chain atoms to adapt to inhibitor (Mahalingam, Wang et al. 2004; Tie, Boross et al. 2004; Tie, Kovalevsky et al. 2007). Here, the inhibitor 1 complexes with PR mutants PR_{R8Q}, PR_{D30N}, PR_{I50V}, PR_{I54M} and PR_{V82A} are analyzed in relation to the PR_{WT}-inhibitor 1 complex and the inhibition values.
2.3 Experimental Section

2.3.1 General

Inhibitor 1 has shown analytical purity of >98% by HPLC (Ghosh, Xu et al. 2010). The structure was confirmed by $^1$H and $^{13}$C NMR spectral analysis, and high resolution mass spectrometry. HRMS (ESI) [M+Na]+ calcd for C30H40N2O9SNa: 627.2352, found: 627.2359.

2.3.2 Protein expression and purification

In order to prevent PR autoproteolysis, a PR clone (Genbank HIVHXB2CG) engineered with five mutations (Q7K, L33I, L63I, C67A and C95A) was used as a template. Mutants (R8Q, D30N, I50V, I54M and V82A) were generated by using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA). The expression, purification and refolding were performed as previously described (Wondrak and Louis 1996; Mahalingam, Louis et al. 2001).

2.3.3 Enzyme kinetics

Kinetic parameters were determined by a fluorescence assay. The fluorogenic substrate was Abz-Thr-Ile-Nle-p-nitro-Phe-Gln-Arg-NH$_2$, where Abz is anthranilic acid and Nle is norleucine (Bachem, King of Prussia, PA, USA), with the sequence derived from the p2/NC cleavage site of the Gag polyprotein. 10 µl protease (final concentration of 70-120 nM) diluted in 98 µl reaction buffer (100 mM Mes, pH 5.6, 400 mM sodium chloride, 1 mM EDTA and 5% glycerol) and 2 µl dimethylsulfoxide or inhibitor (dissolved in dimethylsulfoxide) were incubated at 37 °C for 5 min. The reaction was initialized by adding 90 µl substrate. The reaction was monitored over 5 min in the POLARstar OPTIMA microplate reader at wavelengths of 340 and 420 nm for excitation and emission. Data analysis was performed using the program sigmaplot 9.0 (SPSS Inc., Chicago, IL, USA). $K_m$ and $k_{cat}$ values were obtained by standard data fitting with the Michaelis–Menten equation. The $K_i$ value was obtained from the IC$_{50}$ values estimated from an inhibitor dose–response curve using the equation $K_i = (IC_{50}) [E] / 2 / (1 + [S]/K_m)$, where [E] and [S] are the PR and substrate concentrations.
2.3.4  **Crystallographic Analysis**

Crystals were grown by the hanging drop vapor diffusion method using protein solutions preincubated with inhibitor, which was dissolved in dimethylsulfoxide, in a molar ratio of 1:5-10. The final crystallization drop was 1.6-2 µl with reservoir solution and protein in a 1:1 ratio by volume. Crystalization conditions for different complexes were as follows: 22%-24% saturated Ammonium Sulfate, 130-135 mM Sodium Phosphate, 0.05 M Sodium Citrate, pH 6.1 for PR<sub>R8Q</sub>; 1.46 M NaCl, 0.1 M Sodium Citrate, pH 5.0 for PR<sub>D30N</sub>; 1.26-1.46 M NaCl, 0.06 M Sodium Acetate, pH 5.0-5.4 for PR<sub>I50V</sub>; 0.6-0.93 M NaCl, 0.06 M Sodium Acetate, pH 4.6-5.0 for PR<sub>I54M</sub>; and 10% Ammonium Sulfate, 0.05M Citrate-Phosphate, pH 5.6 for PR<sub>V82A</sub>. The crystals for X-ray data collection were soaked in the reservoir solution with 20-30% glycerol as cryoprotectant for ~1 min and frozen immediately in liquid nitrogen. X-ray data for all the complexes were collected on the SER-CAT beamline at the Advanced Photon Source, Argonne National Laboratory. Data were processed using HKL2000 (Otwinowski and Minor 1997). The structures were solved by molecular replacement using PHASER (McCoy, Grosse-Kunstleve et al. 2005) in the CCP4i suite of programs(Potterton, Briggs et al. 2003). Crystal structures were refined with SHELX97 (Sheldrick 2008). Manual adjustments and rebuilding were performed using the molecular graphics program COOT(Emsley and Cowtan 2004). Figures of the structures were prepared with PYMOL (http://www.pymol.org).

2.4  **Results**

2.4.1  **Compound 1 inhibition of PR<sub>WT</sub> and selected mutants**

The kinetic parameters and inhibition constants (K<sub>i</sub>) of compound 1 for PR<sub>WT</sub> and selected mutants PR<sub>R8Q</sub>, PR<sub>D30N</sub>, PR<sub>I50V</sub>, PR<sub>I54M</sub> and PR<sub>V82A</sub> are shown in Table 1. The catalytic efficiency (k<sub>cat</sub>/K<sub>m</sub>) of PR<sub>V82A</sub> showed 2.7-fold increase, PR<sub>I54M</sub> was essentially identical and PR<sub>R8Q</sub> had a slight decrease relative to PR<sub>WT</sub>. The lowest catalytic efficiency of 10% of PR<sub>WT</sub> was observed for PR<sub>D30N</sub> and PR<sub>I50V</sub>. The relative catalytic efficiency of PR<sub>D30N</sub> measured here is consistent with previous reports using a different substrate (Kovalevsky, Tie et al. 2006). The relative activities of PR<sub>I50V</sub> and PR<sub>V82A</sub> are slightly different from
those reported earlier; however, the trends are identical, with decreased $k_{cat}/K_m$ for PR$_{I50V}$ and increased $k_{cat}/K_m$ for PR$_{V82A}$. Compound 1 showed a range of inhibition of up to 60-fold for the various mutants relative to the wild type enzyme. The inhibition constant of PR$_{R8Q}$ for compound 1 was not significantly different from the value of 0.5 nM for PR$_{WT}$. The PR$_{I54M}$ and PR$_{V82A}$ showed 7- and 6-fold increased $K_i$ for compound 1 relative to PR$_{WT}$. Again, the mutants PR$_{D30N}$ and PR$_{I50V}$ showed the most significant changes with 18- and 60-fold worse inhibition, respectively.

2.4.2 Crystallographic analysis of inhibitor 1 complexes

Crystal structures of PR mutants PR$_{R8Q}$, PR$_{D30N}$, PR$_{I50V}$, PR$_{I54M}$ and PR$_{V82A}$ were obtained in complex with inhibitor 1 and the structures were refined at resolutions of 1.06-1.49 Å. The crystallographic data collection and refinement statistics are summarized in Table 2. All five complexes were determined in space group $P2_12_12$ and refined with anisotropic $B$-factors, solvent molecules, and hydrogen atoms to the R-factors of 13.8-16.3. The asymmetric unit contains one PR dimer of residues labeled 1–99 and 1’–99’ for each monomer. Well defined electron density was observed for the PR residues, as illustrated for the mutated residues in Figure 2. The mutated residues, except for Ala82, show alternative conformations. The side chain of mutated Asn30 in both subunits of PR$_{D30N}$-inhibitor 1 structure has two alternative conformations with relative occupancies of 0.55/0.45. Alternative conformations of the side chains of mutated Met54 and Gln8 with relative occupancies of 0.58/0.42 and 0.55/0.45, respectively, were observed in one subunit in the PR$_{I54M}$ and PR$_{R8Q}$-complexes. Mutated residue Val50 showed alternative conformations for both main chain and side chain atoms in both subunits of PR$_{I50V}$-inhibitor 1 with relative occupancies of 0.7/0.3. Inhibitor 1 has two alternative conformations related by 180° in all five complexes with relative occupancies listed in Table 2.

The residues 48-51/52 at the tip of the flaps show alternative conformations in all the complexes, which was associated with approximately 180° rotation of the peptide bond between Ile50 and Gly51 and alternative conformations of the carbonyl group of Gly48 in both subunits. The electron density map of residues Gly48-Gly52 from the atomic resolution (1.06 Å) structure of PR$_{I55M}$-inhibitor 1 illustrates
clearly the alternative conformations (Figure 3A). Similar disorder in the flaps was observed in the wild type complex with inhibitor 1. However, alternative conformations are unusual for Gly48 in complexes with other inhibitors. Here, in each conformation of the flap residues, the carbonyl oxygen of Gly48 forms several C-H...O interactions with the tris-THF P2 group and Gly48’ forms a C-H...O interaction with the P2’ aniline in the corresponding conformation of inhibitor 1 (Figure 3B). The two conformations of Gly48 may arise from steric hindrance due to the tight fit of the tris-THF group in the S2 subsite.

The mutant dimers superimpose on the PR_{WT}-inhibitor 1 complex (PDB ID: 3OK9) with pairwise root mean square deviations (RMSD) of 0.13-0.24 Å on 198 Cα atoms. Both alternative inhibitor conformations are considered, except for the minor conformation of inhibitor in PR_{D30V}-inhibitor 1, which has the low relative occupancy of 0.3. Hydrogen bond interactions of inhibitor 1 with all five mutants are similar except for variation in interactions of residue 30. The side chain of Asn30/30’ in PR_{D30N} forms a hydrogen bond interaction with the first THF oxygen of inhibitor 1 as observed for the major conformation of inhibitor 1 in the PR_{WT} complex; however, this interaction was not observed for Asp30/Asp30’ in the other four mutants. Interatomic distances for hydrogen bond interactions (O-H...O, N-H...O, etc.) are considered in the range of 2.4–3.4 Å, C-H...O interactions at 3.0–3.7 Å separation, C-H...π interactions for distances of <4.0 Å, and van der Waals contacts when distances are 3.8–4.2 Å, as described previously (Kovalevsky, Liu et al. 2006). Structural changes are described for each mutant separately or in related pairs in the following sections.

2.4.3 *Influence of D30N on PR structure and its interaction with inhibitor 1*

Residue 30 is located at one end of the active site cavity and interacts with the inhibitor, although the side chain frequently has alternate conformations. In PR_{WT}-inhibitor 1, the side chain and main chain amide of the major conformation of Asp30’ form hydrogen bond interactions with the first THF ring of inhibitor 1 (Figure 4A). The main chain of residue 30’ lies in essentially the same position in PR_{D30N}-inhibitor 1 and PR_{WT}-inhibitor 1, thus the hydrogen bond interaction of the main chain amide of Asp30’ with O26 of inhibitor 1 is preserved in the mutant (Figure 4A). In addition, the major conformation of the
side chain (occupancy of 0.55) of residue 30’ was similar in both structures and retained the hydrogen bond interaction with inhibitor. However, the minor conformation (occupancy of 0.45) of the side chain differs in the mutant and the wild type enzyme, especially the position of Oδ1 atom, due to the Cγ movement of approximately 0.9 Å as well as rotation of the side chain (approximately 90°) of Asn30’ compared to Asp30’ of PRWT. As a result, the side chain of Asn30’ in the mutant forms a hydrogen bond with a water molecule, which further interacts with the side chain of Asn88’ as well as the main chain of Thr74’ (Figure 4B). This water also forms hydrogen bond interactions with the main chain and side chain of Thr31’ in both wild type and mutant (not shown in Figure 4B). In the other subunit (1-99), the Asn30 side chain in PRD30N forms a hydrogen bond with the minor conformation of inhibitor 1, which was not observed in PRWT (Figure 4C). The interactions of Asn30 with neighboring residues are similar to those shown in Figure 4C for Asn30’. It seems that the observed 18-fold increase in the inhibition constant of the inhibitor 1 for PRD30N does not arise from changes in the direct interactions between residue 30 and the inhibitor. Instead, the elimination of the negative charge of Asp30 and alterations within an internal network of interactions may provide indirect effects on inhibition. Similar changes in interactions of residues 30 and 88 have been reported in mutants containing D30N and N88D and are also proposed to effect inhibition (Mitsuya, Winters et al. 2006; Kozisek, Bray et al. 2007).

2.4.4 Mutations I54M and V82A produce compensating shifts in the 80’s loop residues

Ile54, which is located in the flap region, does not contact inhibitors directly, but it forms many hydrophobic interactions with surrounding residues, including residues in the 80’s loop (residues 78-82) and Ile50’. In the subunit comprising residues 1-99, Met54 had two alternative conformations as shown in Figure 2. Compared with Ile54 in PRWT, both the major (Figure 5A) and minor (Figure 5B) conformations of Met54 in PRI54M formed one more inter-subunit van der Waals contact with the major and minor conformations of Ile50’. The 80’s loop in PRI54M has shifted to accommodate the larger side chain of Met54 compared with Ile54 in PRWT. Pro79 has shifted away from residue 54 by about 0.8 Å at the Cα atom to maintain their van der Waals interactions. The major conformation of Met54 (relative occupancy of 0.58)
formed the same number of van der Waals contacts with Pro79 (Figure 5A). In addition, it formed one new van der Waals interaction with the major conformation of Thr80. The minor conformation of Met54 (relative occupancy of 0.42) formed the same number of van der Waals contacts with the proline ring (Figure 5B); however, it gained a new C-H...O interaction with the main-chain carbonyl oxygen atom of Pro79 and one more van der Waals interaction with the minor conformation of Thr80 compared with Ile54 in PR<sub>WT</sub>. In the other subunit 1’-99’, Met54’ had a single conformation and formed one less inter-subunit van der Waals contact with both the major (Figure 5C) and minor (Figure 5D) conformations of Ile50 compared with Ile54 in PR<sub>WT</sub>. Unlike subunit 1-99, the 80s loop residue Pro79’ in subunit 1’-99’ exhibited two alternative conformations. A larger shift of approximately 1.1Å was observed for the major conformation of Pro79’ whereas the minor conformation showed an insignificant change of 0.2 Å. Although Met54’ had less van der Waals interactions with the major conformation of Pro79’, it gained a new one with Thr80’ (Figure 5C). The interactions of residue 54’ with the minor conformations of proline were identical for PR<sub>WT</sub> and the mutant (Figure 5D). However, it gained new interactions with the C atoms of Pro79’ and Thr80’. Taken together, there are either two conformations of Met54 or the 80s loop residues to accommodate the large side chain of the mutated Met54. Ile50 exhibits two alternative conformations in all structures as mentioned previously. Met54 in PR<sub>I54M</sub> has a similar number of intersubunit van der Waals contacts with Ile50’ as does Ile54 in PR<sub>WT</sub> (Figures 5A-D). The separation of Pro79 and residue 54 is increased in the mutant so that their van der Waals interaction is retained. The new interactions between Met54 and Thr80 may help maintain the conformation of the rest of the 80’s loop residues despite the shift of Pro79/79’. As shown in Figure 5E, the hydrophobic interactions of Val82 and 82’ with the P1 and P1’ groups of inhibitor 1 are retained in PR<sub>WT</sub> and PR<sub>I54M</sub>. Therefore, the similar catalytic efficiency of PR<sub>I54M</sub> and Pr<sub>WT</sub> may reflect the similar internal contacts of residue 54. Moreover, the hydrogen bond between the major conformation of inhibitor 1 and Asp30 in Pr<sub>WT</sub> was not observed in PR<sub>I54M</sub>, which may contribute to the 7-fold lower inhibition for PR<sub>I54M</sub>.

Val82/82’ has extensive C-H...π interactions with the P1 phenyl group and a van der Waals contact with the P1’ group of inhibitor 1 in Pr<sub>WT</sub> (Figure 5F). Mutation V82A introduces the smaller Ala side
chain, leading to the loss of C-H...π interactions with the P1 phenyl group of inhibitor 1 compared with those in the wild type complex. However, the main chain of Ala82’ has shifted toward the inhibitor by around 0.5 Å compared to the wild type complex, which preserves the hydrophobic interaction with the P1’ of inhibitor 1 (Figure 5F). The interactions of Val82/82’ with the major and minor conformations of the inhibitor were identical, thus only those with the major conformation of the inhibitor 1 are shown. Similar to PR_{I54M}, the hydrogen bond between the major conformation of inhibitor 1 and Asp30 in PR_{WT} was also not observed in PR_{V82A}. The absence of a hydrogen bond plus fewer interactions of Ala82 with the P1 phenyl of inhibitor 1 may be responsible for the decreased inhibitory activity of inhibitor 1 for PR_{V82A}. However, the compensation mechanisms observed in PR_{I54M} and PR_{V82A} may explain why there was only 6-7 fold decrease of the inhibition constants for these two mutants relative to PR_{WT}.

2.4.5 Mutations I50V and R8Q alter the dimer interface and interactions with inhibitor 1

The side chain of Ile50’ in PR_{WT} had intensive interactions with inhibitor 1, including C-H...O and C-H...π interactions (Figure 6A). However, the interactions with the P2’ group of inhibitor were eliminated for the small Val50’ side chain in the mutant PR_{I50V} and only two C-H...O interactions with the sulfonamide oxygen were maintained. Similarly, the two van der Waals interactions between Ile50 and the P2 tris-THF group of inhibitor 1 were lost in the other subunit of PR_{I50V} (Figure 6B). Similar to PR_{I54M} and PR_{V82A}, the hydrogen bond between the major conformation of inhibitor 1 and Asp30 in PR_{WT} was also absent in PR_{I50V}. Taken together, the considerably reduced interactions of mutant PR_{I50V} with inhibitor 1 are consistent with the significantly decreased inhibition (60-fold) for PR_{I50V} compared to the PR_{WT}.

The mutation of Ile50 to Val50 also affects the inter-subunit interactions. In PR_{WT}, the Ile50’ side chain had hydrophobic interactions with the side chains of Ile84 and Ile47, and the same inter-subunit interactions were also observed for Ile50. However, the interactions of Val50’ with both Ile84 and Ile47 are eliminated in the PR_{I50V} mutant as illustrated in Figure 6C. The loss of the interaction between Val50 and Ile47’ was also observed in the other subunit. These findings are consistent with our previously anal-
ysis of PR\textsubscript{I50V} structures (Liu, Boross et al. 2005), and the overall decreased interaction between subunits may account for the 10 fold decrease of its catalytic efficiency as well as likely contributing to the significantly worse inhibition relative to PR\textsubscript{WT}.

In contrast to mutant PR\textsubscript{I50V}, mutation of R8Q has altered the type of interaction rather than eliminated interactions. In the wild type PR, Arg8’ forms an ionic interaction with Asp29 in both subunits (Figure 7A & B). However, this inter-subunit ionic interaction is eliminated in PR\textsubscript{R8Q} (Figure 7C & D). In one subunit of PR\textsubscript{R8Q}, a direct hydrogen bond is formed between the side chains of Gln8 and Asp29’ (Figure 7C). The side chain of Gln8 in PR\textsubscript{R8Q} has shifted away from Arg8 in PR\textsubscript{WT} by approximately 0.9 Å, thus gaining new hydrogen bond interactions with conserved water molecules C and D and a water-mediated interaction with the tris-THF of inhibitor. In the other subunit, Gln8’ has two alternative conformations with relative occupancies of 0.55/0.45 (Figure 7D). The side chain of Gln8’ in the major conformation forms two direct and one water-mediated hydrogen bond with Asp29, whereas that of the minor conformation forms one water-mediated hydrogen bond with Asp29. Overall, direct as well as water-mediated inter-subunit hydrogen bonds between Gln8 and Asp29’ were observed in both subunits, suggesting that these new interactions might compensate for the loss of the inter-subunit ionic interactions in the wild type enzyme (and many other mutants).

The conserved water molecule D also has a hydrogen bond interaction with the oxygen of the third THF ring of inhibitor 1 in both PR\textsubscript{WT} and PR\textsubscript{R8Q} (Figure 7). This water mediates a hydrogen bond interaction between the inhibitor 1 and Gln8/8’, which is not seen for Arg8/8’ in PR\textsubscript{WT} (Figure 7C & D). Moreover, conserved water molecule C mediates an additional hydrogen bond between the major conformation of inhibitor 1 and the side chain of Gln8, which resembles the interaction between the minor conformation of inhibitor 1 and Arg8’ in PR\textsubscript{WT} (Figure 7B).

In summary, although PR\textsubscript{R8Q} has lost the strong inter-subunit ionic interaction between Arg8 and Asp29’, compensation is provided by new direct and water mediated hydrogen bonds. Meanwhile, even though the hydrogen bond between the major conformation of inhibitor 1 and Asp30 in PR\textsubscript{WT} was not seen in PR\textsubscript{R8Q}, the water-mediated hydrogen bond interaction between inhibitor 1 and Gln8/8’ was en-
hanced to some extent. Therefore, there is little absolute change in the interaction of PR\textsubscript{R8Q} with inhibitor 1. In contrast, PR\textsubscript{I50V} showed significant loss of interactions with inhibitor 1. These structural changes explain why inhibitor 1 has 60-fold worse inhibition for PR\textsubscript{I50V} while its inhibition for PR\textsubscript{R8Q} was not affected significantly.

2.5 Discussion and Conclusions

Inhibitor 1 is a novel inhibitor incorporating a unique tris-THF group at P2 to target drug resistant HIV-1 PR mutants. Previously inhibitor 1 was reported to possess potent antiviral activity against wild type HIV-1 and multidrug-resistant strains (Ghosh, Xu et al. 2010). Here, structural and kinetic analyses are described for inhibitor 1 with selected PR mutants. The single mutants showed various effects. Substitution of the smaller side chains in PR\textsubscript{I50V} and PR\textsubscript{V82A} resulted in the loss of their interactions with inhibitor; however, mutation V82A caused a shift of its main chain atoms to compensate for the loss. Mutation to larger side chain in PR\textsubscript{I54M} pushed the Pro79 (80’s loop) away from residue 54 maintaining their interactions; moreover, new interactions were formed between Met54 and Thr80. The elimination of the interactions caused by the change of the residue size in PR\textsubscript{I50V} and PR\textsubscript{V82A} may be related to their worse inhibition ($K_i$) by inhibitor 1. In addition, the mechanisms PR\textsubscript{V82A} and PR\textsubscript{I54M} adopted to compensate for the loss of the interactions may account for the moderate decrease (6–7-fold) in $K_i$ for PR\textsubscript{V82A} and PR\textsubscript{I54M} compared to significant reduction (60-fold) in $K_i$ for PR\textsubscript{I50V}. Moreover, inter-subunit interactions were lost in PR\textsubscript{I50V}, which is expected to contribute to the worse inhibition. Mutations in PR\textsubscript{D30N} and PR\textsubscript{R8Q} eliminated the negative and positive charges of Asp30 and Arg8, respectively. In PR\textsubscript{R8Q}, new hydrogen bond interactions of Gln8 replaced the inter-subunit ionic interaction of Arg8 with Asp29’. Meanwhile, Gln8 formed new water-mediated hydrogen bonds with inhibitor 1, which may explain why inhibitor 1 has similar inhibition for PR\textsubscript{R8Q} and PR\textsubscript{WT}. In terms of hydrogen bonds with inhibitor 1, PR\textsubscript{WT} and all five mutants are similar except for variation in interactions with residue 30 due to the mobility of its side chain. The variation in Asp30/30’ and its interaction with inhibitor did not appear to be a major factor in the $K_i$ for these mutants.
PR\textsubscript{D30N} differs from the other mutants since the major changes are loss of negative charge for Asp30 and altered internal interactions of residue 30/30’ to account for 18-fold decrease of its $K_i$ value for inhibitor 1. D30N mutation is the major mutation associated with resistance to NFV (Johnson, Calvez et al. 2011), which has been suggested to arise from the change of conformational entropy upon inhibitor binding (Kozisek, Bray et al. 2007). Our structural analysis also showed that mutation D30N introduced changes in its interactions with neighboring residues Thr74 and Asn88. In PR\textsubscript{D30N}, the side chain of Asn30 formed water mediated hydrogen bond interactions with both Thr74 and Asn88, whereas, these interactions were absent in PR\textsubscript{WT}-inhibitor 1. Moreover, the proteolytic activity is sensitive to mutations of residues Gly86-Arg87-Asn88 (Ishima, Gong et al.; Ishima, Ghirlando et al. 2001). Considering the positive association of mutation D30N and N88D (Mitsuya, Winters et al. 2006), it would be interesting to study how N88D influences the structure of D30N in complex with this new inhibitor 1.

Compound 1 is an inhibitor designed to fill the S2 binding pocket of PR by incorporating a third THF ring in the P2 group relative to DRV (Figure 1A). As analyzed in its complex with PR\textsubscript{WT} (Figure 7A-B), the oxygen of the third THF ring of inhibitor 1 gains a hydrogen bond interaction with a conserved water (water D in Figure 7A) and helps stabilize the inter-subunit interaction of Arg8’ with Asp29 and other surrounding residues. In the mutant PR\textsubscript{R8Q}, the interaction of the newly incorporated ring of inhibitor 1 with this conserved network was maintained, which is consistent with almost identical inhibition for PR\textsubscript{WT} and PR\textsubscript{R8Q}. Mutation R8Q, however, has rarely been found in resistant clinical isolates. DRV has a $K_i$ value of 6.6 nM for mutant PR\textsubscript{D30N} and up to 18 nM for PR\textsubscript{I50V} consistent with resistance to virus with these mutations (Kovalevsky, Tie et al. 2006). Similarly, our study suggests that viral strains containing D30N or I50V are likely to show resistance to inhibitor 1 given their $K_i$ values of 8.9 and 30.9 nM for PR\textsubscript{D30N} and PR\textsubscript{I50V}, respectively. Inhibitor 1 shows moderate effects on the other two mutants, PR\textsubscript{V82A} and PR\textsubscript{I54M}, similar to their effects with DRV (Tie, Boross et al. 2004; Liu, Kovalevsky et al. 2008). Therefore, mutations D30N, I50V, I54M, and V82A exhibit similar effects on both inhibitors, although inhibitor 1 is less effective than DRV on PR\textsubscript{I50V}. Mutation R8Q exhibits higher sensitivity to compound 1 than other mutations studied here, suggesting that compound 1 may be a good inhibitor against viral strains bearing
R8Q mutation. Drug resistant clinical isolates generally have more than one mutation, however, and previous studies of single and double mutations indicate that the changes in structure and other properties of the respective single mutants may not be preserved in the double mutants (Mahalingam, Boross et al. 2002; Kozisek, Bray et al. 2007). More importantly, the influence of drug resistant mutants on the viral infectivity depends not only on the inhibitor sensitivity, but also on the protease activity, especially for those mutations lacking direct interactions with the inhibitors (Henderson, Lee et al. 2012). Their study showed that mutant PR{sub}I50V had the most negative effect on viral infectivity. Our previous studies suggested that the loss of dimer stability due to I50V mutation was a major contributor to the diminished catalytic activity and inhibition of PR{sub}I50V (Liu, Boross et al. 2005). Mutant PR{sub}D30N also showed ~50% loss of infectivity (Henderson, Lee et al. 2012) in agreement with our previous observation of altered activity on different precursor cleavage sites (Mahalingam, Louis et al. 1999). Therefore, a single mutation may have a variety of effects on inhibition, dimer stability, catalytic activity and viral replication. Finally, since inhibitor 1 possesses the large tris-THF P2 group, our structural analysis suggests it may fit better with the mutants that have an expanded S2/S2’ pocket, such as the extreme multiple mutant PR20 (Agniswamy, Shen et al. 2012).

### 2.6 Acknowledgements

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<th>$K_i$ (nM)</th>
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$^a$ $K_m$ and $k_{cat}$ values previously reported in (Liu, Kovalevsky et al. 2008)

$^b$ $K_m$ and $k_{cat}$ values previously reported in (Chang, Yu et al. 2012)
Table 2.2 Crystallographic Statistics for PR Mutants in Complex with Inhibitor 1

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<td>0.7/0.3</td>
<td>0.53/0.47</td>
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Crystal of $\text{PR}_{\text{D30N}}$ - Inhibitor 1 was obtained by Dr. Johnson Agniswamy; crystallization of $\text{PR}_{\text{I50V}}$ - Inhibitor 1 and $\text{PR}_{\text{I54M}}$ - Inhibitor 1 was done by Chen-Hsiang Shen.
Figure 2.1 The structure of inhibitor compound 1 and PR.

The chemical structures of protease inhibitor compound 1 (A). The structure of HIV-1 PR_{WT}/inhibitor 1. The HIV-1 protease dimer is shown in light blue cartoon representation. The inhibitor 1 and wild-type residues at the mutation sites are indicated by differently colored sticks. The same residues on the two subunits are shown in the same color with only one of them labeled (B).
Figure 2.2 Fo-Fc omit maps for the mutated residues (contoured at 3.0 σ). The magenta sticks indicate the alternative conformations of Asn30, Met54 and Val50.

Figure 2.3 Fo-Fc omit map of flap residues and their interactions with inhibitor 1.
(A) Fo-Fc omit map (contoured at 3.0 σ) for the flap residues Gly48 - Gly52 in complex PRI54M–inhibitor 1 (1.06Å). Carbon atoms are colored cyan and magenta for the major and minor conformations, respectively. (B) Interactions of Gly48/48' with inhibitor 1 in complex PR_{I50V}–inhibitor 1 (0.7/0.3 occupancy). The major and minor conformations of Gly48/48' and the inhibitor 1 are represented by sticks with carbon atoms colored cyan and lines with carbon atoms colored magenta, respectively. C-H…O interactions between the major conformations are indicated by dash-dot lines with distances in Å. C-H…O interactions between the minor conformations are the same but not shown here for clarity.
Figure 2.4 Structural changes of PR\textsubscript{D30N}.
Carbon atoms are colored gray for PR\textsubscript{WT}–inhibitor 1 and cyan for PR\textsubscript{D30N}–inhibitor 1. Hydrogen bond interactions in PR\textsubscript{WT}–inhibitor 1 and PR\textsubscript{D30N}–inhibitor 1 are represented by black and red dashed lines, respectively, with distances in Å. Waters are shown as gray and cyan spheres for PR\textsubscript{WT}–inhibitor 1 and PR\textsubscript{D30N}–inhibitor 1, respectively. Occupancies for alternative conformations are labeled in parentheses for Asp/Asn30’. (A) Interactions of Asp/Asn30’ with the major conformation of inhibitor 1. (B) Interactions of Asp/Asn30’ with the neighboring residues. (C) Interactions of Asp/Asn30 with the minor conformation of inhibitor 1.
**Figure 2.5 Structural changes of PR_{I54M} and PR_{V82A}.**

Carbon atoms are colored gray for PR_{WT}–inhibitor 1 and cyan for PR_{I54M}–inhibitor 1 or PR_{V82A}–inhibitor 1. Interactions are indicated by black lines for PR_{WT}–inhibitor 1 and red lines for the mutants. Van der Waals and C-H...ð interactions are indicated by dotted lines. (A-B) Interactions of the major (A) and minor (B) conformations of Ile/Met54 with Ile50' and 80's loop residues in subunit 1-99. (C-D) Interactions of Ile/Met54' with Ile50 and the major (C) and minor (D) conformation of 80's loop residues in subunit 1'-99'. Relative occupancies for alternative conformations of Met54 and 80's loop residues are shown in parentheses. Red arrows indicate the shifts of the mutants relative to PR_{WT}. (E) Interactions of Val82/82' with inhibitor 1 in PR_{WT} and PR_{I54M}. (F) Interactions of Val/Ala82/82' with inhibitor 1 in PR_{WT} and PR_{V82A}. 
Figure 2.6 Structural changes of PR<sub>I50V</sub>.
(A) Interactions of Ile/Val50 with inhibitor 1. (B) Interactions of Ile/Val50 with inhibitor 1. (C) Inter-subunit interactions of Ile/Val50 with surrounding residues. Carbon atoms are colored gray for PR<sub>WT</sub>–inhibitor 1 and cyan for PR<sub>I50V</sub>–inhibitor 1. Interactions in PR<sub>WT</sub>–inhibitor 1 and PR<sub>I50V</sub>–inhibitor 1 are represented by black lines and red lines, respectively. C-H…O and van der Waals/C-H…δ interactions are indicated by dash-dot lines and dotted lines, respectively, with distances in Å.
Figure 2.7 Structural changes of PR<sub>RSQ</sub>.

Interactions of Arg8/8’ in PR<sub>WT</sub>–inhibitor 1. (C-D) Interactions of Gln8/8’ in PR<sub>RSQ</sub>–inhibitor 1. Carbon atoms are colored gray for PR<sub>WT</sub>–inhibitor 1 and cyan for PR<sub>RSQ</sub>–inhibitor 1. Waters are shown as gray and cyan spheres for PR<sub>WT</sub>–inhibitor 1 and PR<sub>RSQ</sub>–inhibitor 1, respectively. The hydrogen bond interactions are indicated by the dashed lines with distances in Å. Occupancies for alternative conformations of inhibitor 1 and Gln8’ are indicated in parentheses. Hydrogen bond interactions in R<sub>RSQ</sub>–inhibitor 1 are colored red. Interactions between water C and D in all figures (7A-7D) are omitted for clarity.
3 STRUCTURAL AND KINETIC CHANGES IN HIV-1 PROTEASE WITH DRUG-RESISTANT MUTATIONS I47V AND N88D/S

3.1 Abstract

SQV and DRV are two potent clinical inhibitors that were designed to target the wild type PR and its drug resistant mutants, respectively. The crystal structures of PR mutants PR$_{I47V}$, PR$_{N88D/S}$ in complex with DRV and mutants PR$_{I47V}$ and PR$_{N88D}$ in complex with SQV with resolutions of 1.13-1.72 Å were analyzed. Mutation I47V gained more hydrophobic interactions with DRV and SQV. Interestingly, the structural changes did not affect the inhibition for PR$_{I47V}$ (relative $K_i$ is 0.7 and 1 for DRV and SQV, respectively). DRV and SQV showed 8-fold increase in $K_i$ for PR$_{N88D}$ and only small local changes have been observed in the structures suggesting that inhibition is influenced by the gain of a negative charge due to the N88D mutation. DRV showed a modest reduction in $K_i$ for PR$_{N88S}$ probably due to transfer of the distal structural changes to the active site. In sum, this study provided fundamental information for understanding of drug resistance and future design of potential antiviral drugs.

3.2 Introduction

Acquired immunodeficiency syndrome (AIDS) is a life-threatening disease that interferes with the immune system. According to the UNAIDS reports, approximately 35 million people were globally living with HIV/AIDS at the end of 2012 (UNAIDS, 2013). Although there is no effective vaccine available (Girard and Bansal 2008), highly active antiretroviral therapy (HAART), which employs a combination of different antiretroviral drugs, has greatly improved the lives of AIDS patients (Sepkowitz 2001; Brenner, Turner et al. 2002). Antiviral drugs that inhibit the HIV-1 protease (PR) have been an important component of HAART since their first introduction in combination therapy in 1996 (Naggie and Hicks 2010).
PR cleaves the viral precursor polypeptides Gag and Gag-Pol into the mature structural and enzymatic proteins (Darke, Nutt et al. 1988; Oroszlan and Luftig 1990). Inhibition of this PR leads to immature noninfectious virus (Gottlinger, Sodroski et al. 1989; Louis, Ishima et al. 2007). Thus PR is an effective target for antiviral drugs. HIV-1 PR is catalytically active as a homodimer of two subunits designated as residues 1-99 and 1'-99'. It is a member of the aspartic protease family and contains conserved catalytic triplet Asp25-Thr26-Gly27 as a key part of active site cavity. Early studies also identified a second conserved triad Gly86-Arg87-Asn88 in HIV-1 PR (Pearl and Taylor 1987; Rao, Erickson et al. 1991). Whereas Asp25-Thr26-Gly27 is conserved in all aspartic acid proteases, Gly86-Arg87-Asn88 in the α-helix is only shared by retroviral proteases (Pearl and Taylor 1987; Rao, Erickson et al. 1991; Louis, Weber et al. 2000). The dimer interface, including the catalytic Asp25 from each subunit, interactions of Arg8 with Asp29 and Arg87' from the other subunit (Weber 1990), the flexible flaps comprising residues 45 to 55 and the four-stranded beta sheet formed by the termini, is also critical for PR activity and stability (Mahalingam, Louis et al. 1999; Weber and Agniswamy 2009).

The most severe challenge to the long-term efficacy of protease inhibitors (PIs) in HAART therapy is the frequent emergence of drug-resistant mutants of PR due to the infidelity of the HIV reverse transcriptase (Condra, Schleif et al. 1995; Richman, Morton et al. 2004; Mehellou and De Clercq 2010; Menendez-Arias 2010). These mutants show much lower affinity for PIs while their critical PR function in viral replication is still maintained (Miller 2001). Extensive structural and kinetic studies of HIV-1 PR and its drug resistant mutants have been investigated in the absence or presence of substrates or inhibitors in order to reveal the molecular mechanisms of drug resistance and to contribute to the structure-based design of HIV-1 PR inhibitors (Weber and Agniswamy 2009). Up to date, there are nine clinical PIs approved for AIDS therapy. Saquinavir (SQV, figure 1A), which was the first clinical inhibitors, was designed to bind tightly in the active site cavity of the wild type enzyme. The binding affinity of SQV, however, can be readily lowered by mutations in the PR. The most recently approved drug darunavir (DRV, figure 1A) was designed to target drug resistant PR mutants by incorporating more hydrogen bond interactions with PR main chain atoms. DRV contains two tetrahydrofuran groups (bis-THF) at the P2 position...
tion and has demonstrated high potency on resistant viral infections in the laboratory (Koh, Nakata et al. 2003; Surleraux, Tahri et al. 2005) and in clinic (de Meyer, Vangeneugden et al. 2008).

Mutation I47V is associated commonly with DRV resistance in the clinic (De Meyer, Lathouwers et al. 2009). Residue 47 lies in the flap region; therefore, mutation of this residue may affect the conformation and flexibility of the flap, thus influencing the binding affinity of the substrates or inhibitors. Moreover, the side chain of Ile47 (green sticks in Figure1B) points towards the active site cavity and provides several van der Waals interactions to stabilize the inhibitor binding to PR. Mutation I47V was found to confer resistance to almost all FDA approved drugs including DRV, with the only exception of SQV. Therefore, we investigated how PR bearing the I47V mutation (designated PR_{I47V}) exhibits different effects on DRV and SQV.

Mutations of Asn88 to Asp or Ser are also common in drug resistance. Similar to the catalytic triad Asp25-Thr26-Gly27, Gly86-Arg87-Asn88 triad is also conserved in retroviral proteases (Pearl and Taylor 1987; Rao, Erickson et al. 1991; Louis, Weber et al. 2000). Two aspartic acids at residue 25 from two subunits form the central active site and are essential for enzyme activity. The mutation of Asp25 leads to complete loss of the enzyme’s ability to hydrolyze the substrate peptide bonds (Kohl, Emini et al. 1988; Seelmeier, Schmidt et al. 1988). Although the Gly86-Arg87-Asn88 triad does not directly contact the substrate or inhibitor, it is located close to the active site loop and dimer interface. The inter-subunit salt-bridge between R87 and D29 is crucial for dimer stability of the mature PR (Weber 1990); It has been shown that both the D29N and R87K mutants increased the dimer dissociation constant ($K_d$) by several orders of magnitude(Ishima, Torchia et al. 2007). The increased dimer dissociation caused by R87K mutants then drastically diminished the catalytic activity of HIV-1 PR (Ishima, Torchia et al. 2007). The role of the G86 residue in dimer formation and catalytic activity was also studied by the same group (Ishima, Gong et al. 2010). They have demonstrated that G86 mutants also exhibited extremely low catalytic activities. However, G86 mutants reduced the catalytic activities without significantly increasing the dimer dissociation constants. Structural studies by NMR and X-ray crystallography indicated that G86 mutants have conformational changes in G86 site and these changes also influence the active site region, thus hav-
ing an impact on substrate/inhibitor binding. While these residues in conserved region either severely affect the catalytic activity (such as G86 and R87) or completely diminish the enzyme activity (Asp25), mutations on residue 88 was the only one selected in drug resistance. Therefore, it is very important to address whether and how N88 influences the structure and activity of HIV-1 PR.

Residue Asn88 is located in the alpha helix (labeled yellow in Figure 1B) and its side chain interacts with the residues Asp29, Thr31 and Thr74. Mutation from N88 to D/S may change these interactions, which may be related to decreased catalytic activity for PR with the N88D mutation (Mahalingam, Louis et al. 1999). In the complex of PR_{D30N} with the investigational antiviral inhibitor GRL-0519, the side chain of Asn30 formed water mediated hydrogen bond interactions with both Thr74 and Asn88 (Zhang, Wang et al. 2013). Thus, residue 88 may interact indirectly with the inhibitor binding site through interactions with Asp29, Thr31 and Asp30. Moreover, the occurrence of D30N facilitates selection of mutations N88D and L90M during evolution of drug resistance (Mitsuya, Winters et al. 2006). Mutation N88D is usually found in the treatment of nelfinavir (NFV) as an accessory mutation, which compensates for the loss of catalytic activity due to the major change of D30N (Parera, Fernandez et al. 2007). Mutations of N88 are not associated with DRV resistance, however, mutation N88S confers resistance to SQV (Rhee, Taylor et al. 2010). Hence, we are also interested in comparing the structural and kinetic effects of N88S and N88D on two typical clinical drugs SQV and DRV.

### 3.3 Experimental Section

#### 3.3.1 Protein expression and purification

In order to prevent PR autoproteolysis, a PR clone (Genbank HIVXB2CG) engineered with five mutations (Q7K, L33I, L63I, C67A and C95A) was used as a template. Mutants (I47V, N88S and N88D) were generated by using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA). The expression, purification and refolding were performed as previously described (Wondrak and Louis 1996; Mahalingam, Louis et al. 2001).
3.3.2 **Enzyme kinetics**

Kinetic parameters were determined by a fluorescence assay. The fluorogenic substrate was Abz-Thr-Ile-Nle-p-nitro-Phe-Gln-Arg-NH$_2$, where Abz is anthranilic acid and Nle is norleucine (Bachem, King of Prussia, PA, USA), with the sequence derived from the p2/NC cleavage site of the Gag polyprotein. 10 µl protease (final concentration of 70-120 nM) diluted in 98 µl reaction buffer (100 mM Mes, pH 5.6, 400 mM sodium chloride, 1 mM EDTA and 5% glycerol) and 2 µl dimethylsulfoxide or inhibitor (dissolved in dimethylsulfoxide) were incubated at 37 °C for 5 min. The reaction was initialized by adding 90 µl substrate. The reaction was monitored over 5 min in the POLARstar OPTIMA microplate reader at wavelengths of 340 and 420 nm for excitation and emission. Data analysis was performed using the program sigmaplot 9.0 (SPSS Inc., Chicago, IL, USA). Km and kcat values were obtained by standard data fitting with the Michaelis–Menten equation. The Ki value was obtained from the IC$_{50}$ values estimated from an inhibitor dose–response curve using the equation $Ki = (IC_{50} \times [E]/2)/(1 + [S]/Km)$, where [E] and [S] are the PR and substrate concentrations.

3.3.3 **Urea denaturation assay**

The urea denaturation effect was measured using a fluorescence assay. The PR activity was measured in 200 µl solution: 50 µl urea with the final concentration ranging from 0 M to 4.0 M; 10 µl PR at the final concentration of 20 nM to 35 nM; 90 µl anthranylyl substrate at the final concentration of 72 µM and 50 µl denaturation buffer (200 mM MES, 800 mM sodium chloride, 2 mM EDTA, 10% glycerol at pH 5.6). The UC$_{50}$ values were determined by plotting the initial velocities against increasing urea concentration using SigmaPlot 8.02.

3.3.4 **Crystallographic Analysis**

Crystals were grown by the hanging drop vapor diffusion method using protein solutions preincubated with inhibitor, which was dissolved in dimethylsulfoxide, in a molar ratio of 1:5. The final crystallization drop was 2 µl with reservoir solution and protein in a 1:1 ratio by volume. Crystallization
conditions for different complexes were as follows: 0.41M sodium chloride, 0.1M Citrate phosphate, pH 5.8 for PR\textsubscript{I47V}-DRV; 1.35M sodium chloride, 0.1M Citrate phosphate, pH 6.0 for PR\textsubscript{I47V}-SQV; 0.4M sodium chloride, 0.1M sodium acetate, pH 4.4 PR\textsubscript{N88S}-DRV; 0.26 sodium chloride, 0.1M Tris-HCl, pH 6.5 for PR\textsubscript{N88D}-SQV; 1.5M Sodium Chloride, 0.1M Citrate-Phosphate, pH 5.6 for PR\textsubscript{N88D}-DRV. The crystals for X-ray data collection were soaked in the reservoir solution with 20-30% glycerol as cryoprotectant for 1 min and frozen immediately in liquid nitrogen. X-ray data for all the complexes were collected on the SER-CAT beamline at the Advanced Photon Source, Argonne National Laboratory. Data were processed using HKL2000(Otwinowski and Minor 1997). The structures were solved by molecular replacement using PHASER(McCoy, Grosse-Kunstleve et al. 2005) in the CCP4i suite of programs(Potterton, Briggs et al. 2003). Crystal structures were refined with SHELX97(Sheldrick 2008). Manual adjustments and rebuilding were performed using the molecular graphics program COOT(Emsley and Cowtan 2004). Figures of the structures were prepared with PYMOL (http://www.pymol.org).

3.4 Results

3.4.1 DRV and SQV inhibition of catalytic activity of PR\textsubscript{WT} and drug resistant mutants

The kinetic parameters and inhibition constants ($K_i$) of DRV and SQV for PR\textsubscript{WT} and its drug resistant mutants PR\textsubscript{I47V}, PR\textsubscript{N88S} and PR\textsubscript{N88D} are shown in Table 1. The catalytic efficiency ($k_{cat}/K_m$) of PR\textsubscript{I47V} was essentially identical (1.2-fold) or slightly increased relative to PR\textsubscript{WT}. Both PR\textsubscript{N88D} and PR\textsubscript{N88S} had a slight decrease (0.6 and 0.7-fold, respectively) in the catalytic efficiency relative to PR\textsubscript{WT}. While PR\textsubscript{I47V} and PR\textsubscript{N88S} showed slight decrease (0.7, 0.3-fold respectively) in their inhibition constants ($K_i$) for DRV relative to PR\textsubscript{WT}, PR\textsubscript{N88D} exhibits approximately 8-fold increase for both DRV and SQV. The $K_i$ of PR\textsubscript{N88S} for SQV also increased, but only 3-fold, relative to PR\textsubscript{WT}. Mutant PR\textsubscript{I47V} has the same $K_i$ for SQV as PR\textsubscript{WT}.

The dimer stability of PR\textsubscript{WT} as well as three PR mutants PR\textsubscript{I47V}, PR\textsubscript{N88S} and PR\textsubscript{N88D} was measured by using urea denaturation assay. UC$_{50}$ value, which is the concentration of denaturing urea when the PR activity drops to 50% of the initial value, was obtained to evaluate the stability of PR\textsubscript{WT} and its mutants.
The UCI50 value of PRI47V was increased by approximately 40% of the wild type value, while those of PRN88D and PRN88S were decreased by approximately 50%. These results suggest that the mutation of flap residue Ile47 increased the PR dimer stability, whereas the mutation Asn88 decreased the PR dimer stability.

3.4.2 Crystallographic analysis

Crystals of PR mutants PRI47V, PRN88D and PRN88S in complex with DRV and mutants PRI47V and PRN88D in complex with SQV were obtained by using the hanging drop vapor diffusion method. The crystal structures were refined at resolutions of 1.13-1.72 Å as shown in Table 3. All five complexes were determined in space group P21212 and refined with solvent molecules to the R-factors of 12.5-21.7. All complexes except PRN88D_DRV were also refined with anisotropic B-factors and hydrogen atoms. The asymmetric unit contains one PR dimer composed of residues labeled 1–99 and 1’–99’ for the two monomers. The mutated residues in five complexes were clearly shown by the electron density maps illustrated in Figure 2. The mutated residues I47V in complex PRI47V–SQV and N88D in complex PRN88D_DRV showed one conformation. Two alternative conformations of the mutated residue in one of the subunits were observed for the other three complexes, as illustrated in Figure 2. DRV and SQV have two alternative conformations related by 180° in all five complexes with relative occupancies listed in Table 3. Both alternative conformations are considered in PRI47V–DRVcomplex. Structural changes were analyzed by comparing the mutants with wild type PR. The mutant dimers superimposed on the PRWT_DRV or PRWT–SQV complexes with pairwise root mean square deviations (RMSD) of 0.13-0.51 Å on 198 Ca atoms. Slightly larger differences were seen for the surface loops of residues 37-42/37’-42’ in the SQV complexes, which is probably due to the different space groups since PRWT–SQV was determined in space group P21212 (Mahalingam, Wang et al. 2004).

3.4.3 Comparison of PRI47V_DRV and PRWT_DRV

In the PRWT_DRV, Ile47 is located in the hydrophobic pocket and interacts with Val32, Leu76, and residues in the flap region, including Lys45, Ile54 and Val56, and also forms intersubunit interactions
with Ile50' (Figure 3A). Similar to Ile47, Ile47’ in another subunit interacted with its surrounding residues Val32’, Leu76’, lys45’, Ile54’, Val56’ as well as Ile50. Furthermore, it showed a C-H…O interaction with one alternative conformation of the side chain of Asp30’ (Figure 3B). The I47V mutation to a smaller side chain might be expected to eliminate interactions with inhibitor. However, the side chain of Val47 showed two alternative conformations and partially compensated for the lost interactions. One side chain conformation (with occupancy of 0.58) of Val47 has lost van der Waals contacts with Leu76, Val32 and Lys45, nevertheless, the other conformation (with occupancy of 0.42) of Val47 retained interaction with Leu76 (Figure 3C). In addition, the intersubunit interaction between residue 47 and Ile50’ is preserved due to a small shift of the main chain. Similar to Ile47, Ile47’ in another subunit interacted with its surrounding residues Val32’, Leu76’, lys45’, Ile54’, Val56’ as well as Ile50. Furthermore, it showed a C-H…O interaction with one alternative conformation of the side chain of Asp30’ (Figure 3B). The I47V mutation to a smaller side chain might be expected to eliminate interactions with inhibitor. However, the side chain of Val47 showed two alternative conformations and partially compensated for the lost interactions. One side chain conformation (with occupancy of 0.58) of Val47 has lost van der Waals contacts with Leu76, Val32 and Lys45, nevertheless, the other conformation (with occupancy of 0.42) of Val47 retained interaction with Leu76 (Figure 3C). In addition, the intersubunit interaction between residue 47 and Ile50’ is preserved due to a small shift of the main chain. Val47’ in the other subunit also lost interactions with neighboring residues Asp30’, Val32’, Lys45’ and Ile54’ compared to the wild type complex, while the intersubunit contact with Ile50 is preserved (Figure 3D). The shift in main chain position as well as rotation of the side chain of Val47/47’ also leads to shorter C-H…π interactions with DRV (Figure 3C-D). The hydrogen bond interactions of DRV are essentially identical in the mutant and wild type enzyme. Therefore, the major structural change is the loss of internal hydrophobic interactions of residue 47 in the mutant, which may tend to increase flap mobility and hence lower the affinity for inhibitor. This effect would be consistent with the association of I47V with resistance to DRV (Johnson, Calvez et al. 2013).
3.4.4  Comparison of PR<sub>I47V</sub>_SQV and PR<sub>WT</sub>_SQV

Ile47 in PR<sub>WT</sub>_SQV has hydrophobic interactions with Val32, Leu76, and flap residues Lys45, Ile54 and Val56 similar to the complex with DRV, however there is no van der Waals contact with Ile50' from the other subunit (Figure 4A). In addition, Ile47 has a hydrophobic interaction with SQV. In the other subunit, Ile47’ formed similar interactions (Figure 4B). In the mutant PR<sub>I47V</sub>_SQV, Val47 has lost interactions with Val32 compared with those in the wild type protease complex, as shown in Figure 4C. In addition, the closest interatomic distance between Val47 and SQV is slightly out of the range for good van der Waals interactions. The interactions of Val47’ are the same as those of Val47 except for an additional CH...O interaction with Asp30’ (Figure 4D). Analysis of SQV interactions with residues of PR<sub>I47V</sub> showed that hydrogen bond interactions were conserved in the mutant and wild type enzyme. However, the 80s’ loop of PR<sub>I47V</sub>_SQV was shifted towards SQV so that the hydroxyl group of Thr80 forms a new CH...O interaction with SQV. The flap region in the other subunit also shifted so that new CH...O interactions were formed between SQV and residues Gly48’, Val47’ and Asp30’ (Figure 4E). Taken together, SQV in complex PR<sub>I47V</sub>_SQV has lost a hydrophobic contact with mutated Val47 in one of the subunits; however, it has gained several CH-O interactions with the enzyme. The observed gain of interactions of this mutant with SQV is in excellent agreement with clinical results suggesting that mutation I47V does not alter susceptibility for SQV (Johnson, Calvez et al. 2013).

3.4.5  Structural change of PR<sub>N88D</sub>_DRV and PR<sub>N88S</sub>_DRV relative to PR<sub>WT</sub>_DRV

Asn88 has important connections with residues in the active site cavity and the dimer interface. In the wild type structure of PR<sub>WT</sub>_DRV, Asn88 forms hydrogen bond interactions with Thr74, Thr31 and Asp29 in both of the subunits (Figure 5A-B), while Asp29 has an intersubunit ionic interaction with Arg87’. In the mutant PR<sub>N88D</sub>_DRV, Asp88 in one subunit formed interactions resembling those in PR<sub>WT</sub>_DRV (Figure 5C). However, the direct hydrogen bond between Asp88’ and Thr74’ is lost in the other subunit, instead, the side chain of Asp88’ rotates to allow entry of a water molecule forming water-mediated interactions linking Asp88’ with Thr74’ and Thr31’ (Figure 5D-E). Superimposition of the two
complexes showed that the carbonyl group of Asp88' rotates 12° towards Thr91' side chain and forms a new hydrogen bond (Figure 5E). The side chain of Asp88' also swings 20° towards Gly86' forming another new hydrogen bond. Compared to PR<sub>WT</sub>_DRV, the main chain of Thr74' in PR<sub>N88D</sub>_DRV shifts 1Å away from Asp88'. The concerted shift of residues 73' to 75' is further transmitted to the outer strand of residues Gln58'-Ile62' to preserve the β-sheet (Figure 5E). In addition to these structural changes and introduction of a water molecule near Asn88', PR<sub>N88D</sub>_DRV has lost a water-mediated hydrogen bond observed between DRV and side chain of Asp30 in the wild type complex. These structural changes are assumed to contribute to the loss of catalytic activity and increased sensitivity to urea observed for this mutant; however, they do not appear to produce large changes in the interaction with DRV. The increased <i>K<sub>i</sub></i> measured for DRV inhibition may be related to the addition of a negative charge for N88D.

In the other mutant PR<sub>N88S</sub>_DRV, the side chain of Ser88 is rotated 19° outwards from Thr74 and Thr31 and forms a direct hydrogen bond with Asp30 side chain. The additional space allows introduction of water–mediated hydrogen bonds that compensate for the loss of direct hydrogen bonds with Thr74 and Thr31 (Figure 6A). In the other subunit, Ser88' has two alternative conformations, one rotates 15° outwards and the other rotates 19° inwards (Figure 6B). The inward movement of the second alternate conformation enables the hydroxyl group of Ser88' to form hydrogen bonds with Thr31' resembling those observed for the amino group of Asn88' in the wild type enzyme (Figure 6B). However, the first alternate conformation of Ser88' allows two waters to occupy the space between Ser88' and Thr74', Thr31' and form a network of interactions among these residues (Figure 6C). Similar to PR<sub>WT</sub>_DRV, the hydrogen bond interaction between the main chain atoms of residue 88 and Asp29 in PR<sub>N88S</sub>_DRV was observed in both subunits. Presumably, the shorter serine side chain due to the N88S mutation and the introduction of internal water is responsible for the lower activity and stability observed for PR<sub>N88S</sub>, while these structural changes produce little change in inhibitor of DRV, in agreement with the lack of association of N88D mutation with resistance to DRV.
3.4.6 Comparison of PR\textsubscript{N88D-SQV} and PR\textsubscript{WT-SQV}

In PR\textsubscript{WT-SQV}, Asn88 formed direct hydrogen bond interactions with Thr74 and Thr31. Thr74 is also linked with Asp30 through waters (Figure 7A-B). Compared to wild type enzyme, the side chain of Asp88 in PR\textsubscript{N88D-SQV} shifts 18° towards Asp30 and loses the hydrogen bond with the carbonyl oxygen of Thr74 even though it is compensated by a water-mediated interaction (Figure 7C). There is a change in the position but not the linkage of one of two waters which mediate the interactions among residues 88, 30, 31 and 74. The direct hydrogen bond interaction of residue 88 with Thr31 is retained in the mutant and wild type enzyme. The C\alpha of Asp88’ in the other subunit of PR\textsubscript{N88D-SQV} shifts approximately 0.6 Å, thus eliminating the direct hydrogen interaction with Thr74’. The side chain of Asp88’ has two alternative conformations rotated 43° and 113°, respectively, as shown in Figure 7D. While one conformation of Asp88’ forms a water-mediated hydrogen bond interaction with Thr74’, the other exhibits no interaction with Thr74’. As for the DRV complexes, the structural changes due to N88D involve changes in water-mediated interactions around residues 88, 30-31 and 74, which are likely due in part to the introduction of a negative charge in the mutant with Asp88.

3.5 Discussion

SQV and DRV are two potent clinical inhibitors that were designed to target the wild type protease and its drug resistant mutants, respectively. Here, we performed structural and kinetic analysis of SQV and DRV with single substitution mutants PR\textsubscript{I47V} and PR\textsubscript{N88D/S}. Substitution of the smaller side chain in PR\textsubscript{I47V} is expected decrease its interactions with surrounding residues. However, mutation I47V in PR\textsubscript{I47V-DRV} maintained most of its hydrophobic interactions with surrounding residues (Figure 3). Meanwhile, the shifts of the 80s’ loop and the flap region observed in the structure of PR\textsubscript{I47V-SQV} enables the formation of new CH…O interactions with SQV as well as between Val47’ and Asp30’ (Figure 4E). The alteration of the interaction between PR\textsubscript{I47V} and SQV does not affect the inhibitory activity.
of SQV (relative $K_i$=1), which agrees with the clinical observation showing little effect of the I47V mutation on susceptibility to SQV.

Our previous study of HIV-1 protease in complexes with its reaction intermediates demonstrated that the substitution of Ile47 by the shorter side chain residue Val47 completely eliminates its internal hydrophobic contacts with neighboring residues including Val32, Val56, Leu76, Ile54 and Ile50’ from the other subunit (Shen, Tie et al. 2012). This alteration caused by the mutation I47V differs from what we observed here in complexes with clinical inhibitors DRV and SQV. Comparison of three superimposed structures (product complex [PDB ID: 4FLG]/with DRV/with SQV) of PR$_{I47V}$ with PR$_{WT}$ indicates that the retained interactions of I47V with adjacent residues in complexes PR$_{I47V}$-DRV and PR$_{I47V}$-SQV are mainly caused by rotation of its side chain or/and significant main chain shift (Figure 4E). The presence of the inhibitor in the complexes may induce these conformational changes. Indeed, DRV formed new interactions with mutated residue Val47 in complex PR$_{I47V}$-DRV, and SQV gained interactions with Gly48’ and Thr80 in PR$_{I47V}$-SQV. In addition, the side chain flexible conformations of Asp30 may contribute to the main chain shift of Val47’ in PR$_{I47V}$.

The structure and inhibition of HIV PR with the mutation I47A was described (Saskova, Kozisek et al. 2008). Substitution of Ile47 by Ala is rarely seen in HIV-positive patients and emerges with the prolonged treatment of lopinavir (LPV). They showed that SQV exhibited 2-fold increase in $K_i$ value for the mutant with I47A relative to the wild type enzyme; however, no structure was reported for PR$_{I47V}$ with SQV. The structure of PR$_{I47V}$-LPV shows a loss of interaction with LPV. This result is more extreme than the alteration induced by I47V, partly due to the further decrease of the side chain.

Replacement of Asn at residue 88 with Asp (N88D) or Ser (N88S) is frequently found with prolonged exposure to NFV. In PR$_{N88D}$ complex with DRV (Figure5), even though the side chain of mutated Asp88 in both subunits rotates towards Gly86’, in one subunit there is no significant change in its interaction with surrounding residues (Figure5A, C), whereas in the other subunit the direct hydrogen bond between Asp88’ and Thr74’ is replaced by a water-mediated interaction and coordinated shifts of the adjacent beta strands (Figure 5B, D, E). In PR$_{N88S}$ complex with DRV, the flexibility and the presence of two
alternative conformations of Ser88’ not only enable a direct hydrogen bond with Asp30 side chain, but also comparable connections with Thr74 and Thr31 as seen in the wild type complex. However, the linkage of Ser88’ with Asp30’ eliminates the hydrogen bond interactions between Asp30’ and DRV. In PR<sub>N88D</sub>-SQV (Figure7), the most significant alteration between the wild type and mutant is the loss of hydrogen bond between Asp88 and Thr47.

Consistent with the unliganded structure of PR<sub>N88D</sub> (Zhang, Wang et al. 2013), both structures of PR<sub>N88D</sub> in complexes with DRV and SQV showed the presence of water-mediated hydrogen bond between Asp88 and Thr74 in the mutant instead of the direct hydrogen bond seen in the wild type enzyme (Figure5&Figure7). However, the water-mediated connection of Asp88 with Asp30 reported in the unliganded structure of PR<sub>N88D</sub> was observed in PR<sub>N88D</sub>-SQV (Figure7C-D) but not in PR<sub>N88D</sub>-DRV (Figure5C-D). This configuration was not observed in the native unliganded structure of PR<sub>N88D</sub> but found in PR<sub>WT</sub>-SQV (Figure5A-B). Therefore, we cannot conclude that the presence of this connection is due to the mutation or affected by the presence/type of the inhibitors. Even though Asp30 does not have a hydrogen bond interaction with Asp88, DRV still lost its interaction with Asp30 in PR<sub>N88D</sub>-DRV. This may be related to the single conformation of Asp30 in the mutant. Our kinetic analysis suggested that DRV and SQV exhibit the same inhibitory effects on PR<sub>N88D</sub> (relative Ki =8). It seems that the interaction of Asp30 with inhibitor does not have significant effect on the $K_i$ for some mutants as we reported previously (Zhang, Wang et al. 2013).

Similar to the unliganded structure of PR<sub>N88S</sub> (Zhang, Wang et al. 2013), the direct hydrogen bonds of residue 88 with Thr74 and Thr31 in the complex of PR<sub>N88S</sub>-DRV are lost in both subunits. In one of the subunits of PR<sub>N88S</sub>-DRV, their connections are replaced by water-mediated hydrogen bonds (Figure6A). In another subunit, Ser88’ exhibited two alternative conformations, one conformation formed direct hydrogen bonds with Thr31’, thus filling the role of Asn88’ ND2 in wild type protease (Figure6B). The other conformation of Ser88’ interacted with Thr74’ and Thr31’ through two waters which fill the bigger cavity created by mutation N88S (Figure6C). Therefore, the interactions among residue88, thr74 and Thr31 do not alter as much as in the unliganded structure of PR<sub>N88S</sub>. Compared to the wild type
unliganded PR, unliganded PR_{N88S} gained a direct hydrogen bond interaction between Ser88 and Asp30. In complex PR_{N88S}-DRV, Ser88 gained direct hydrogen bond with Asp30 in one subunit (Figure 6A) and water mediated hydrogen bond in another one (Figure 6C). However, this did not affect the interaction between Asp30 and DRV. The inhibitory effect of DRV for PR_{N88S} (relative $K_i = 0.3$) is much higher than for PR_{N88D} (relative $K_i = 8$). However, it is hard to address how exactly these structural alterations influence the inhibitory effects, maybe indirectly through the network of interactions connecting residue 88 with Asp29-Asp30 in the active site cavity.

3.6 Acknowledgements

This work was supported in part by the Georgia State University Molecular Basis of Disease Fellowships (H.Z. and X.Y.), the Georgia State University Research Program Enhancement Award in Bioinformatics (X.Y.). Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number U01GM062920. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. X-ray data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) beamlines at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at www.ser-cat.org/members.html. Use of the Advanced Photon Source was supported by the US Department of Energy, Basic Energy Sciences, Office of Science, under Contract No. W-31-109-Eng-38.
### Table 3.1 Kinetic Parameters for Substrate Hydrolysis and Inhibition of DRV and SQV.

<table>
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<tr>
<th>Protease</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$</th>
<th>Relative</th>
<th>DRV $K_i$ (nM)</th>
<th>Relative $K_i$</th>
<th>SQV $K_i$ (nM)</th>
<th>Relative $K_i$</th>
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<td>PR</td>
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<td>194±23</td>
<td>7.4±1.2</td>
<td>1.0</td>
<td>0.58±0.10</td>
<td>1</td>
<td>0.42±0.07</td>
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<td>0.42±0.04</td>
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<td>3.14±0.33</td>
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*a $K_m$ and $k_{cat}$ values previously reported in Liu et al.2008

### Table 3.2 Urea Denaturation

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<td>N88S-DRV</td>
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<td>P2₁2₁2</td>
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<td>6.9(44.2)</td>
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<td>Completeness (%) overall (final shell)</td>
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<td>Average B-factors (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
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<td>Main-chain atoms</td>
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<td>Side-chain atoms</td>
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<tr>
<td>Solvent</td>
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<tr>
<td>Inhibitor occupancy (%)</td>
<td>0.58/0.42</td>
<td>0.65/0.35</td>
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Crystallization and structure refinement was partially done by Xiaxia Yu.
Figure 3.1 Structures of PR and inhibitor SQV and DRV.
(A) Chemical structures of protease inhibitor SQV and DRV. (B) The structure of HIV-1 PR<sub>WT</sub>/DRV. The HIV-1 protease dimer is shown in light blue cartoon representation. Ile 47 is colored cyan and DRV is indicated by yellow sticks. Two conserved triads Asp25-Thr26-Gly27 and Gly86-Arg87-Asn88 are colored red and green, respectively. The same residues on the two subunits are shown in the same color.
Figure 3.2 Fo-Fc omit maps for the mutated residues. Fo-Fc omit maps are contoured at 3.0σ. The alternative conformations are indicated by the magenta sticks.
Figure 3.3 Structural changes of PR_{I47V} in complex with DRV.
Carbon atoms are colored gray for PR_{WT} and green for PR_{I47V}, respectively. Carbon atoms of DRV in both complexes are colored yellow. Van der Waals interactions and C−H···π interactions are indicated by black square dotted lines with distances in Å. Interatomic distance > 4Å are shown in red round dotted lines to indicate the absence of C−H···π interactions. (A-B) Interactions of Ile47/Ile47’ with surrounding residues in PR_{WT,DRV}. (C-D) Interactions of Val47/Val47’ with surrounding residues in PR_{I47V,DRV}.
Figure 3.4 Structural changes of PR\textsubscript{I47V} in complex with SQV.
Carbon atoms are colored gray for PR\textsubscript{WT} and green for PR\textsubscript{I47V}. Carbon atoms are colored yellow for SQV in complex PR\textsubscript{I47V-SQV} and gray for SQV in complex PR\textsubscript{WT-SQV}. C–H···O and van der Waals interactions are indicated by black dash–dotted lines and square dotted lines, respectively, with distances in Å. Interatomic distance > 4.3Å are shown in red round dotted lines to indicate the absence of van der Waals interactions. (A,B) Interactions of Ile47 with surrounding residues in complex PR\textsubscript{WT-SQV}. (C,D) Interactions of Val47' with surrounding residues in complex PR\textsubscript{I47V-SQV}. (E) PR\textsubscript{I47V-SQV} superimposed with PR\textsubscript{WT-SQV}. The C–H···O interactions of SQV with neighboring residues. The atom shifts in Å are indicated by red arrows.
Figure 3.5 Structural changes of PR_{N88D} DRV.
Hydrogen bond interactions of Asn88/88' (A-B), Asp88/88'(C-E) with surrounding residues are indicated by the dashed lines with distances in Å. Carbon atoms are colored gray for PRWT and green for PRN88D. Water molecules are shown as red spheres. Hydrogen bond interactions of Asn88/88' and Asp88/88' superimposed in figure 5E are indicated by black and red dashed lines, respectively. The rotation of the chain is also labeled in degree. Backbone of residues Gln58'-Ile62' and Gly73'-Val75' are represented by lines.
Figure 3.6 Structural changes of PR\textsubscript{N88S\_DRV}.
PR\textsubscript{N88S\_DRV} is superimposed with PR\textsubscript{WT\_DRV}. Hydrogen bond interactions of Ser88 (A) /88’(B-C) with surrounding residues are indicated by the dashed lines with distances in Å, whereas hydrogen bond interactions in PR\textsubscript{WT\_DRV} refer to Figure 5 A-B. The hydrogen bond interactions of the two conformations of Ser88’ with the surrounding residues are indicated in B and C, respectively. Carbon atoms are colored gray for PR\textsubscript{WT} and green for PR\textsubscript{N88S}. Water molecules are shown as red spheres for PR\textsubscript{N88S} and gray for PR\textsubscript{WT}. The hydrogen bond interaction between two waters in panel C was omitted for clarity. The rotation of the side chain of residue 88 in PR\textsubscript{N88S} relative to wild type is indicated in degrees.

Figure 3.7 Structural changes of PR\textsubscript{N88D\_SQV}.
The hydrogen bond interactions of Asn88/Asn88’ (A-B) and Asp88/88’(C-D) with surrounding residues are indicated by the dashed lines with distances in Å. Carbon atoms are colored gray for PR\textsubscript{WT} and green for PR\textsubscript{N88D}. Water molecules are shown as red spheres. The shift is labeled by arrow with distances in Å and the rotation of the chain is also labeled in degree.
4 OVERALL SUMMARY AND DISCUSSION

HIV-1 PR is an effective target for treatment of HIV/AIDS. The employment of PIs in antiviral therapy has made successful progress in reducing mortality and improving the life quality of AIDS patients. However, the long-term efficacy of PIs is challenged by the rapid emergence of drug-resistant mutants of PR. To understand the underlying mechanisms of drug resistance, structures and activities of HIV-1 PR and its drug resistant mutants have been extensively studied. Various effects have been observed for these mutations located in different regions of PR. Generally, the mutations in the inhibitor binding sites result in the loss of their interactions with inhibitors, thus the PR variants show reduced binding affinity for the inhibitors. In contrast, the mutations located in the distal region lead to subtle and indirect effects on the structure and activity of PR. Mutation of residues located in the flap region may contribute to dimer stability of PR and its interactions with inhibitors.

In this study, PR mutants PR_{R8Q}, PR_{D30N}, PR_{I50V}, PR_{I54M}, PR_{V82A}, PR_{I47V}, and PR_{N88IDS} bearing single substitutions have been investigated by crystallography and kinetics. These mutations, with the exception of R8Q, are common in drug resistant clinical isolates. The structural and kinetic effects of mutations from different regions including active site (PR_{D30N}, PR_{I50V}, PR_{V82A}), flap region (PR_{I50V}, PR_{I54M}) and dimer interface (PR_{R8Q}, PR_{I50V}) were analyzed with a novel potent inhibitor GRL-0519. PR variants bearing mutations at the active site (PR_{D30N}, PR_{I50V}) had lowest binding affinity with inhibitors. V82A also is located in the active site cavity but the alteration in its interactions with inhibitor was moderate due to the compensatory effects from the main chain shift. Flap residues may also be components of the active site cavity as well as the dimer interface, which is exemplified by Ile50. Due to its critical role in these important regions, mutation of these residues may influence the flap conformation, inhibitor binding affinity and dimer stability. This explains why mutant PR_{I50V} had the most severe effects: 60-fold decrease in its inhibition relative to PR_{WT} and 50% loss of dimer stability (Liu, Boross et al. 2005; Zhang, Wang et al. 2013). Even though PR_{I54M} also has a mutated flap residue, Ile54 does not interact with inhibitor or form inter-subunit interactions. The moderate effect of I54M is also due to the compensating shifts in the 80’s
loop residues. The most significant effect of mutations in dimer interface is the alteration in inter-subunit interaction and consequently dimer stability (Mahalingam, Louis et al. 1999). Our study demonstrated that the inhibitory activity of GRL-0519 on PR_{R8Q} is similar to that of PR_{WT}. However, the maintained binding affinity with the inhibitor may be attributed to the large S2 group of GRL-0519 and its water mediated hydrogen bond interactions with Gln8.

The effects of mutations occurring in the flap region (I47V) and a distal region (N88D/S) of PR were analyzed with inhibitors SQV and DRV. Compared to mutation I50V, which lost the extensive interactions formed with inhibitor GRL-0519 in wild type PR, I47V did not cause significant change in its interaction with inhibitors SQV and DRV: either loss or gain of one van der Waals interaction with the inhibitor. Similarly, there was no significant alteration observed in inter-subunit interaction in mutant PR_{I47V}. This may in part due to the shift of main chain and rotation of side chain of I47V. Therefore, mutation of flap residues may exhibit various effects, depending on how much they are involved in its interaction with inhibitor and dimer stability. Residue 88 is located in the distal region of PR. Asn88 is part of the triplet Gly-Arg-Asn that is conserved in retroviral PRs (Pearl and Taylor 1987; Louis, Weber et al. 2000). It has been reported that the effects caused by mutations on distal regions can be transferred to the active site and affect the properties of PR indirectly (Liu, Boross et al. 2005). Indeed, structural analysis of PR_{N88S}-DRV showed that Ser88’ formed a new hydrogen bond with Asp30’ side chain. The linkage of Asp30’ with Ser88’ makes Asp30’ move away from DRV, thus eliminating the hydrogen bond interactions between Asp30’ and DRV. However, the loss of interaction between Asp30 and DRV did not cause much change on the inhibitory effects of DRV partially due to the flexibility of Asp30. Our previous study has shown that the variation in Asp30/30’ and its interaction with inhibitor did not appear to be a major factor in the $K_i$ for the mutants (Zhang, Wang et al. 2013).

GRL-0519 is an inhibitor designed to fill the S2 binding pocket of PR by incorporating a third THF ring in the P2 group relative to DRV. Our investigation of the inhibitory effects of GRL-0519 on mutants PR_{D30N}, PR_{I50V}, PR_{I54M}, and PR_{V82A} was compared with previous inhibitory study of DRV on the same mutation. It seems that mutations D30N, I50V, I54M, and V82A exhibit similar effects on both in-
hibitors, although GRL-0519 is less effective than DRV on PR_{I50V} (Tie, Boross et al. 2004; Kovalevsky, Tie et al. 2006; Liu, Kovalevsky et al. 2008; Zhang, Wang et al. 2013). However, the large tris-THF group at P2 of GRL-0519 provides a good fit with the mutants that have an expanded S2/S2’ pocket, such as the extreme multiple mutant PR20 (Agniswamy, Shen et al. 2012). The structural and kinetic effects of SQV and DRV on the same drug resistant mutants were also compared and no significant difference of was observed.

In this study, only PR mutants bearing single substitutions have been investigated by crystallography and kinetics. Drug resistant clinical isolates generally have more than one mutation, however, and previous studies of single and double mutations indicate that the changes in structure of the respective single mutants are usually conserved in the double mutants while other properties may not be preserved (Mahalingam, Boross et al. 2002; Kozisek, Bray et al. 2007). It is more difficult to apply the information from single mutation to multiple mutations or extreme mutations such as clinically derived PR20 (Louis, Aniana et al. 2011; Agniswamy, Shen et al. 2012). In addition, the development of mutation is so fast and our studies are always behind. It is always better to prevent the selection of the mutations than to combat the endless mutations that have already emerged. One way to limit the development of drug resistance is to have the inhibitor to fill the whole active site. A good start for this strategy is the development of the antiviral inhibitor GRL-0519, which possesses a large tris-THF group at P2. Our most recent study showed that the large tris-THF group fits the expended hydrophobic S2 pocket of PR20 much better than others (Agniswamy, Shen et al. 2013). In addition, targeting the flexible flap region may be an efficient way to limit the emergence of resistance. First, the flap residues are components of both active site and dimer interface. Additionally, the flexibility of the flap is very closely related to inhibitor binding affinity. Finally, it has been reported that the potent inhibitor DRV has a second binding site on the flap regions (Kovalevsky, Liu et al. 2006). In sum, inhibitors that are designed to have more polar interactions with the flap residues and have large P2/P2’ could be highly efficient to limit the emergence of viral resistance.
REFERENCES


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**APPENDICES**

### Appendix A: List of crystal structures

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Appendix B

2 Appendix B.1 Canonical D1 Receptor Signal Transduction Cascade Potassium Current in the Pyloric Network by the Cell Specific Dopamine Modulation of the Transient

Hongmei Zhang1, Edmund W. Rodgers1, Wulf-Deiter C. Krenz1, Merry C. Clark2, Deborah J. Baro1

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Abstract

Dopamine (DA) modulates motor behaviors in many systems through well conserved transduction cascades. To better understand how DA modulates motor activity from the cellular to network levels, we study a small circuit. The pyloric network located within the stomatogastric ganglion (STG) of the spiny lobster, Panulirus interruptus, consists of 14 identifiable neurons, and an extensive literature exists on DA modulation of cellular properties and circuit output. We demonstrate that DA consistently altered cellular properties but had variable effects on circuit output. In keeping with previous work, two electrode voltage clamp (TEVC) showed that in >90% of the cells examined, DA excited the lateral pyloric (LP) and inhibited the pyloric dilator (PD) neurons, in part by decreasing and increasing the peak transient potassium current (IA), respectively. However, DA had variable effects on cycle frequency. We previously demonstrated that PD expressed type 2 DA receptors (D2Rs), but not type 1 (D1Rs). Here we show that LP expressed somatodendritic D1Rs, but not D2Rs. LP D1Rs were concentrated near synapses and coupled with Gs-AC-cAMP-PKA to modulate IA. We characterized specific antagonists in a heterologous expression system: Flupenthixol had a ten-fold higher affinity for D1Rs while metoclopramide specifically antagonized D2Rs. Flupenthixol and metoclopramide completely blocked DA-induced changes in LP and PD IA, respectively. Metoclopramide always blocked DA-induced changes in cycle frequency while

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flupenthixol had variable effects. Antagonist application to a spontaneously active network had subtle and variable effects, and suggested that endogenous levels of DA continuously modulated the spontaneous rhythmic activity produced by a network.

**Keywords:** *Panulirus interruptus*; neuromodulation; dopamine receptors; signal transduction; cAMP; shal channel; pharmacology

**Introduction**

The crustacean pyloric network is a powerful model for studying neuromodulation of rhythmic behaviors. All the major cells and their circuit connections are known. Many projection and sensory neurons that modulate the network have been defined (Stein, DeLong et al. 2007; Blitz, White et al. 2008; Daur, Nadim et al. 2009; DeLong, Beenhakker et al. 2009; Hedrich, Smarandache et al. 2009), and the neuromodulatory effects of monoamines and peptides on this circuit have been extensively studied (Nusbaum and Beenhakker 2002; Marder and Bucher 2006; Stein 2009).

Bath applied DA alters circuit output by differentially modulating pyloric neuron synaptic and intrinsic firing properties (Harris-Warrick, Johnson et al. 1998). This is partially mediated by DA modulation of several ion channels (Harris-Warrick, Coniglio et al. 1995; Harris-Warrick, Coniglio et al. 1995; Cleland and Selverston 1997; Harris-Warrick, Johnson et al. 1998; Kloppenburg, Levini et al. 1999; Kloppenburg, Zipfel et al. 2000; Peck, Nakanishi et al. 2001; Johnson, Kloppenburg et al. 2003; Peck, Gaier et al. 2006). In this manuscript, we focus on DA modulation of $I_A$, which helps determine the rate of post-inhibitory rebound and spike frequency in pyloric neurons and influences pyloric cycle frequency (Tierney and Harris-Warrick 1992).

DA can change the voltage dependence of $I_A$ in a cell specific manner. For example, bath-applied DA shifts $I_A$ voltage dependence negatively in PD neurons (Kloppenburg, Levini et al. 1999) and positively in LP neurons (Harris-Warrick, Coniglio et al. 1995). Differences in DA transduction cascades may underpin these opposing effects, since shal channels mediate $I_A$ and appear to have the same subcellular
distribution in both identified cell types (Baro, Coniglio et al. 1996; Baro, Levini et al. 1997; Baro, Ayali et al. 2000).

DA acts on several highly conserved DA receptors (DARs) that belong to the G protein-coupled receptor (GPCR) superfamily. All DARs can be classified into two types based on their structure and signaling mechanisms: D1Rs and D2Rs. DARs can signal through many pathways including G protein dependent and independent cascades. In the canonical pathways, D1Rs increase and D2Rs decrease cAMP (Neve, Seamans et al. 2004). The two spiny lobster D1Rs, D1αPan and D1βPan, and the single D2R, D2αPan, signal through canonical pathways when overexpressed in HEK cells (Clark and Baro 2006; Clark and Baro 2007) and in native stomatogastric membrane preparations (Clark, Khan et al. 2008). In addition, lobster DARs can signal through conserved, non-canonical pathways. In STG membrane preparations D1αPan can couple with Gq as well as Gs, and D2αPan can couple with PLCβ via Gβγi/o in HEK cells.

We have recently shown that PD neurons express D2Rs, but not D1Rs (Oginsky, Rodgers et al. 2010). These receptors are concentrated in perisynaptic regions and appear to receive volume DA transmissions. In the present study, we test the hypothesis that DA has opposing effects on PD and LP neurons, because LP neurons express D1Rs while PD neurons express D2Rs. Further, the effects of endogenous DA and individual DARs on spontaneously active pyloric circuits have never been investigated. As a first step toward understanding the innate role of DA in generating a rhythmic motor output, here we define DAR antagonists and apply them to spontaneously active preparations.

**Materials and Methods**

**Drugs**

Drugs were all purchased from Sigma (St. Louis, MO) except Rp-cAMP, tetrodotoxin (TTX) and H-89 (Tocris Bioscience, Bristol, UK).

**Animals**

California spiny lobsters, *Panulirus Interruptus*, were purchased from Don Tomlinson Commercial Fishing (San Diego, CA, USA) and kept in aerated and filtered artificial saltwater tanks.
**Dissection and cell identification**

Lobsters were cold-anaesthetized for at least 30 min and the Stomatogastric Nervous System (STNS) was dissected as previously described (Bierman and Tobin 2009; Tobin and Bierman 2009). The STNS was pinned in a Sylgard-lined dish and continuously superfused with *Panulirus* (P.) saline (in mM): 479 NaCl, 12.8 KCl, 13.7 CaCl$_2$, 39 Na$_2$SO$_4$, 10 MgSO$_4$, 2 Glucose, 4.99 HEPES, 5 TES; pH 7.4.

Cells were identified using standard intracellular and extracellular recording techniques as previously described (Harris-Warrick, Coniglio et al. 1995; Harris-Warrick, Coniglio et al. 1995). Neuronal activity was monitored with intracellular somatic recordings using 20–40 MΩ glass microelectrodes filled with 3 M KCl and Axoclamp 2B or 900A amplifiers (Axon Instruments, Foster City, CA). Extracellular recordings of identified motoneurons were obtained using a differential AC amplifier (A-M Systems, Everett, WA) and stainless steel pin electrodes. Neurons were identified by their distinct waveforms, the timing of their voltage oscillations, and correlation of spikes on the extracellular and intracellular recordings.

**LP dye fills**

The identified LP neuron was filled with a lysine fixable, dextran coupled Texas Red fluorophore that was impermeable to gap junctions (Molecular Probes), as previously described (Clark, Khan et al. 2008). A 1% solution of the fluorophore in 0.2 mol/L KCl was pressure injected (8-20 psi, 200ms pulse, 0.05 Hz) using a 8–15 MΩ glass microelectrode and a PicoSpritzerIII (General Valve / Parker Hannifin). The fluorophore was injected until the cell became dark purple (typically 15-25 min, depending on the microelectrodes resistance).

**Single cell RT-PCR**

The single cell RT-PCR protocol consisted of three steps: cell isolation, reverse transcription and PCR.

Cell Isolation: After cell identification, the ganglion was incubated at room temperature with 1.2 mg/ml of collagenase type IA (Sigma) or 2 mg/ml collagenase/Dispase (Sigma) in P. Saline without Ca$^{2+}$ until the cells were loosened from the ganglion (typically 10 min). The identified LP neuron was then physically isolated from the STG using mouth suction and a fire polished micropipette that was previous-
ly baked at 75°C to ensure that it was RNase free. Using a dissecting microscope, the LP neuron was blown into the cap of a microcentrifuge tube. The cap containing the neuron was replaced on the tube and immediately stored on dry ice.

Reverse Transcription: Individual LP neurons were processed for reverse transcription using a cells-to-cDNA kit (Ambion) as described in (Oginsky, Rodgers et al. 2010)

PCR: 4.5 µl aliquots of the reverse transcribed cDNA from a given LP neuron served as templates for four PCRs, each containing a different primer set specific for one of the three lobster DARs or α-tubulin (Oginsky, Rodgers et al. 2010). Advantage Taq polymerase (Clontech) was used according to the manufacturer’s instructions. PCR products were run on a 10% polyacrylamide gel (70 V for 1:30 hrs) and stained with ethidium bromide for 15min. The bands were visualized under UV light using a Fluochem 8800 gel imager (Alpha Innotech, San Leandro, CA).

Immunohistochemistry (IHC)

The LP neuron was filled with the Texas Red fluorophore, and the STNS was bathed in L-15 culture media (1 package of L-15 compounds dissolved in 2 L of the P. saline + 100 unit/ml penicillin-streptomycin + 0.05 mg/ml neomycin) for 4-24 hrs at room temperature to allow the fluorophore to diffuse. The preparation was then fixed and DAR protein distributions were determined using wholemount STG preparations in IHC experiments, followed by confocal microscopy. The IHC protocol was as previously described (Baro, Ayali et al. 2000; Clark, Dever et al. 2004). The primary, affinity purified antibodies against the three lobster DARs (D1αPan, D1βPan and D2αPan) and their respective specificities were previously described (Clark, Khan et al. 2008; Oginsky, Rodgers et al. 2010). Data were acquired with a LSM510 Confocal Laser Scanning Microscope from Carl Zeiss Microimaging (Oberkochen, Germany).

cAMP assays in a heterologous expression system

Receptors were transiently expressed in human embryonic kidney (HEK) or COS7 cells as previously described (Spitzer, Cymbalyuk et al. 2008). DA induced changes in cAMP were measured using an ELISA assay kit (Assay Designs), as previously described (Clark and Baro 2006; Clark and Baro 2007;
Spitzer, Cymbalyuk et al. 2008; Spitzer, Edwards et al. 2008). The IC$_{50}$s of flupenthixol and metoclopramide were determined for each lobster DAR by exposing cells that over-expressed a given receptor to DA, or DA plus varying concentrations of a given antagonist. Data were analyzed with Prism (GraphPad) and Excel (Microsoft) software.

**TEVC**

$I_A$ was analyzed with TEVC as previously described using an Axoclamp 2B or 900A amplifier and Clampex 8.2 software (Baro, Levini et al. 1997). Briefly, the desheathed STN and STG were surrounded by two Vaseline wells. The STG was continuously superfused at room temperature with P. saline using a Rainin Dynamax peristaltic pump. Experiments on LP were performed at ambient temperature (20-22°C); and those on PD at 16-18°C. After cell identification, descending inputs were removed with a sucrose block (30% sucrose in distilled water plus 0.3423g/ml, 0.005% Fast Green) applied to the well surrounding the single input nerve, stomatogastric nerve (stn), for 1 hr. These are termed, deafferented preparations. Glutamatergic synaptic inputs were blocked with picrotoxin ($10^{-6}$M). All known voltage dependent ion channels except $I_A$ were blocked with TTX ($10^{-7}$M), TEA, ($2\times10^{-2}$M), CsCl ($5\times10^{-3}$M) and CdCl$_2$ ($2\times10^{-4}$M).

The LP neuron was impaled with low resistance (5–9 MΩ) microelectrodes filled with 3 M KCl. Cells were held at -50mV. Protocols to determine the voltage of activation consisted of a series of 10mV steps from -50mV to +60mV, with or without a hyperpolarizing prepulse to -90mV. $I_A$ was further isolated by digitally subtracting recordings obtained without prepulses from those with prepulses. Steady state inactivation was recorded at +20mV following a series of hyperpolarizing steps from -110mV to -20mV with 10mV increment. In most experiments drugs were continuously superfused into the STG well. However, in order to save on costs, during Rp-cAMP application, perfusion pumps were stopped after the drug was applied to the bath and the bath volume had been replaced at least 5 times. Pumps were started again for washout. No change in holding currents was observed during this process. In all experiments the concentration of DA was 10µM, except the Rp-cAMP experiments where DA was reduced to 5µM to save on costs. In some experiments, the order of application was reversed to show that the drug had the same ef-
fect regardless of whether or not it was preceded by DA application (e.g., antagonist alone and antagonist +DA applications were before the DA application).

Data were analyzed with Clampfit v.8.2 (Axon Instruments), Prism v.4 and 5 (Graphpad) and Excel (Microsoft). After digital subtraction, peak currents measured at each voltage step were converted into conductance using $G = \frac{I_{\text{peak}}}{(V - E_k)}$, assuming $E_k = -86\,\text{mV}$. The calculated conductance and the corresponding voltage were then used to construct conductance-voltage plots. The plots were fit with a first-order Boltzmann equation to obtain the maximal conductance ($G_{\text{max}}$), the apparent voltage of half activation ($V_{1/2}^{\text{activation}}$) and voltage of half inactivation ($V_{1/2}^{\text{inactivation}}$).

Statistical analyses:

Unless otherwise indicated, data are shown as mean ± SEM. Student’s t-tests were performed with Excel software. One-way repeated measures ANOVA and curve fitting were performed with GraphPad Prism software. Tukey’s post hoc tests were performed where appropriate. Statistical significance was determined as $p<0.05$.

Results

Expression of DARs in the LP neurons

In order to understand how DA effects changes in LP $I_A$, we first defined LP DAR expression. LP neurons were electrophysiologically identified, physically isolated and processed for single cell RT-PCR. In spiny lobsters, there are two D1Rs, $D_1^{\alpha\text{Pan}}$ and $D_1^{\beta\text{Pan}}$, and one D2R, $D_2^{\alpha\text{Pan}}$ (Clark and Baro 2006; Clark and Baro 2007). Each LP was tested for the presence of all three lobster DAR, as well as $\alpha$-tubulin transcripts (Figure 1). As shown in Table 1, while all LP neurons expressed $\alpha$-tubulin, DAR expression appeared to vary amongst preparations.

To determine whether or not protein expression similarly varied, we performed IHC experiments on STG wholemount preparations, each containing a dye-filled LP neuron. Three custom made, affinity purified antibodies, each specific for one of the three lobster DARs, were used in conjunction with confo-
cal microscopy as previously described (Clark, Khan et al. 2008; Oginsky, Rodgers et al. 2010). Whereas DARs may be localized to multiple subcellular compartments, shal channels are exclusively targeted to the somatodendritic compartment (Baro, Ayali et al. 2000). Overlapping 1µm confocal optical sections throughout the somatodendritic compartment of a given LP neuron were therefore examined for the presence of the receptor. The data suggested that receptor expression in the somatodendritic compartment was consistent across preparations: D_{1αPan} and D_{1βPan} receptors were always observed in LP neurons, but D_{2αPan} receptors were never detected (n=5 for each DAR; Figure 2).

Similar to our findings for PD D2Rs (Clark, Khan et al. 2008; Oginsky, Rodgers et al. 2010), LP D1Rs appeared to be located in endomembrane structures and transported to synaptic varicosities. Both D_{1αPan} and D_{1βPan} receptors were observed in LP perinuclear vesicles (Figure 2A, B &F) although vesicular staining was less obvious for D_{1αPan} receptors. D1Rs were also detected in cytoplasmic transport vesicles in primary and higher order neurites (Figure 2C, D &G). Careful examination of the optical sections suggested that receptors were not associated with the plasma membrane in these structures. Receptors were most highly concentrated in varicosities along, or at the terminals of fine neurites (Figure 2C, E, H &I), which are known to represent synaptic structures (King 1976; King 1976). D_{1αPan} and D_{1βPan} receptors often appeared to be in the plasma membrane of synaptic varicosities, as there was no rim of red cytoplasm surrounding receptor immunoreactivity (Figure 2E &I).

Our previous studies suggested that D1Rs may be expressed in glial cells (Oginsky, Rodgers et al. 2010). Figure 2 illustrates that D_{1βPan} receptors were highly expressed in the processes of glial and/or other support cells in the STG. It was not clear from our IHC experiments whether or not D_{1αPan} and D_{2αPan} receptors were expressed in glial cells. They are not obviously in the membrane of glial somata, as are shal channels (Baro, Ayali et al. 2000). If these DARs are expressed in glia, they have a punctate distribution and cannot be differentiated from neuronal staining.

In sum, the LP neuron consistently expressed D1Rs, but not D2Rs in the somatodendritic compartment. Receptor expression patterns in other compartments are unknown. LP DAR transcript expres-
sion patterns appeared to vary, but it is not clear whether this is a biological reality or technical artifact (see Discussion).

*LP D1Rs couple with *I*<sub>a</sub>* through a *Gs-AC-PKA* but not *Gq* cascade

We previously showed that D<sub>1αPan</sub> and D<sub>1βPan</sub> couple with Gs, and D<sub>1αPan</sub> can also couple with Gq in STNS membrane preparations (Clark, Khan et al. 2008). If DA acts exclusively through LP D1Rs, then DA effects on LP may be mediated by Gs and/or Gq transduction cascades. A pharmacological dissection of the DA induced transduction cascades modulating LP *I*<sub>a</sub> was therefore performed using TEVC.

Consistent with previous reports (Harris-Warrick, Coniglio et al. 1995), a 10min bath application of 10<sup>-5</sup> M DA reversibly decreased the peak *I*<sub>a</sub> evoked by a depolarizing test pulse following a hyperpolarizing prepulse to remove all channel inactivation (Figure 3A). DA induced positive shifts in *I*<sub>a</sub> voltage dependence (Fig. 3B). Regardless of their initial voltage dependencies, most cells showed similar depolarizing shifts in their apparent voltages of half activation (3.4 ± 0.3 mV) and inactivation (3.9 ± 0.3 mV, Figure 3C). It is noteworthy that < 10% of the cells examined did not respond to DA, or responded with a smaller negative shift. Whereas DA also decreased *I*<sub>a</sub>G<sub>max</sub>, this reduction was not reversible, and will not be considered further here.

We first tested whether DA decreased LP *I*<sub>a</sub> via a *Gs* cascade. Adenylyl cyclase (AC) converts ATP to cAMP, and Gs can stimulate AC activity. We asked whether or not forskolin, an AC activator, could mimic and, at saturating concentrations, occlude the effects of DA on LP *I*<sub>a</sub>. A 10 min 10<sup>-5</sup> M DA application was followed by a 30 min washout, and forskolin (50 µM) was applied for 10 min followed by a 10 min application of DA (10<sup>-5</sup> M) plus forskolin (50 µM). LP *I*<sub>a</sub> was recorded at the end of each drug application and wash. Forskolin shifted the LP *I*<sub>a</sub> *V*<sub>1/2</sub> activation by 6.2 ± 0.8 mV and the *V*<sub>1/2</sub> inactivation by 3.8 ± 0.7 mV (n=5, Figure 4). These shifts were significantly different from control (p<0.01), but not from the shift induced by DA alone. Thus, forskolin mimicked the effects of DA on LP *I*<sub>a</sub>. Moreover, addition of 10<sup>-5</sup> M DA to preparations that previously received 50 µM forskolin did not produce fur-
ther significant shifts in the voltage dependence (P > 0.05). Thus, saturating levels of forskolin can oc-
clude the effects of DA on LP $I_A$. In order to exclude the possibility that forskolin acted directly on shal-
channels, rather than on AC, we examined the effect of 1, 9-dideoxyforskolin (dd-forskolin), a structural
analogue of forskolin that does not activate AC. We found that dd-forskolin had no significant effects on
LP $I_A$ (n=3). Taken together, these data suggest that DA shifts the voltage dependence of $I_A$ at least par-
tially through AC.

If LP D1Rs couple with Gs to stimulate AC, then an increase in cAMP should mimic the effects
of DA on LP $I_A$. We tested this hypothesis by bath applying varying concentrations of the membrane
permeable cAMP analogue, 8-Bromo cAMP. We found that cAMP and DA produced similar dose-
dependent changes in the voltage dependence of LP $I_A$ (Figure 5).

PKA is one of the major effectors of cAMP. In order to test whether PKA was involved in the
signaling pathway that mediated DA’s effect on LP $I_A$, we examined whether or not PKA inhibitors could
block DA’s actions. In this experiment the preparation was exposed to $10^{-5}$ M DA for 10min, followed by
a 30 min wash. The PKA inhibitor, H-89 (20µM), was then applied for 10 min at 20µM followed by an
application of H-89 plus DA. $I_A$ was recorded prior to DA application, at the end of each of the drug
treatment, and after the 30 min wash. The voltages of half activation and inactivation were determined for
each condition, and the change from baseline was plotted in Figure 6, where baseline is the $V_{1/2}$ prior to
drug treatment (DA) or after the wash (H89, H89+DA). The data demonstrate that H-89 inhibited the DA
effect. A 10 min bath application of $10^{-5}$ M DA caused a significant shift in $V_{1/2}$ activation (Figure 6A, 6.2
± 0.3 mV, p < 0.001) and inactivation (Figure 6B, 6.2 ± 0.5 mV, p<0.001) that could be washed out.
However, application of DA plus H89 had no significant effect on $I_A$ voltage dependence. Because H89
can also inhibit other kinases, such as PKG, we repeated the experiment with a specific and expensive
PKA inhibitor, Rp-cAMP. To save on the cost of the inhibitor in this experiment, the concentration of DA
was reduced to 5 µM. Whereas 5 µM DA induced a significant and reversible shift in $I_A$ $V_{1/2}$ activation
(Figure 6C, p<0.001) and inactivation (Figure 6D, p<0.01), Rp-cAMP blocked this effect. Interestingly,
Rp-cAMP itself significantly altered $I_A V_{1/2}$ inactivation ($P < 0.05$). This might suggest that PKA constitutive modulates the $I_A V_{1/2}$ inactivation. Indeed, all of the PKA blockers shifted $I_A$ voltage dependence in the opposite direction to DA, but in most cases the changes were not statistically significant. Taken together, our data suggest that the Gαs-AC-cAMP-PKA signaling pathway mediates DA modulation of $I_A$ in the LP neuron, consistent with the fact that LP expresses somatodendritic D1Rs, but not D2Rs.

As mentioned above, D1αPan receptors also couple with Gq in STNS membrane preparations. Therefore, we wanted to test whether DA also modulated LP $I_A$ via a Gq cascade. Phospholipase Cβ (PLCβ) is the downstream target of activated Gαq subunits. Using the aforementioned experimental paradigm, we asked whether ET-18-OCH₃, a PLCβ inhibitor, could block DA modulation of LP $I_A$. Addition of ET-18-OCH₃ did not prevent the DA induced shift in voltage dependence (Figure 7). Therefore, we conclude that DA modulates LP $I_A$ through a Gs, but not Gq cascade.

*Flupenthixol and metoclopramide are specific antagonists for lobster D1 and D2 receptors, respectively*

To further confirm that DA modulates $I_A$ exclusively through D1Rs, we sought to obtain antagonists specific for lobster D1Rs and D2Rs. It is well established that monoamine receptor pharmacology is not conserved between vertebrate and invertebrate receptors (Blenau and Baumann 2001; Tierney 2001; Spitzer, Cymbalyuk et al. 2008; Spitzer, Edwards et al. 2008). We therefore performed a preliminary screen using a cadre of drugs ($n = 1$ per drug) and found candidate antagonists specific for lobster D1Rs (flupenthixol) and D2Rs (metoclopramide). The effects of these drugs were characterized in detail using a transient, heterologous expression system and assays for DA induced changes in cAMP concentration in the presence and absence of flupenthixol and metoclopramide.

Consistent with previous studies (Clark and Baro 2006; Clark and Baro 2007), DA altered cAMP levels in both D1R and D2R expressing HEK cells. Flupenthixol blocked the DA induced cAMP increase in D1αPan (flupenthixol IC₅₀=2.2×10⁻⁸ M; n=3) and D1βPan (flupenthixol IC₅₀=1.4×10⁻⁹ M; n=6) expressing HEK cells (figure 8); however, metoclopramide did not antagonize the DA effect on these cells even at
concentrations of 100µM (n=4 for each DAR). On the other hand, DA induced changes in cAMP were prevented by metoclopramide in D_{2αPan} expressing HEK cells (metoclopramide IC_{50}=1.2×10^{-7} M; n=3). In addition, flupenthixol also antagonized DA effects on D_{2αPan} expressing HEK cells (IC_{50}=2.3×10^{-7} M; n=5). These data indicate that metoclopramide is a specific antagonist for lobster D2R signaling and flupenthixol has a ten-fold higher affinity for D1Rs than D2Rs expressed in HEK cells.

**DAR specific antagonists confirm that DA acts on LP D1Rs and PD D2Rs**

Figure 2 suggests that LP neurons exclusively express D1Rs in their somatodendritic compartment, and we previously showed that PD neurons exclusively express D2Rs (Oginsky, Rodgers et al. 2010). DA modulates I_{A} in opposing directions in PD and LP, largely by shifting the voltage dependence of activation positively (LP, Figure 3) or negatively (Kloppenburg, Levini et al. 1999). If DA acts on LP and PD neurons exclusively through D1Rs and D2Rs, then flupenthixol should block DA modulation of LP but not PD I_{A}, and metoclopramide should block DA modulation of PD but not LP I_{A}. Figure 9 illustrates that this is indeed the case. Using TEVC, we measured the DA induced change in the V_{1/2} activation in LP and PD in the presence and absence of each antagonist. In these experiments, DA (10^{-5} M) was bath applied for 10 min and washed for 30 min; then, 10µM antagonist was bath applied for 5 min, followed by a 10 min application of antagonist plus DA followed by a 30min wash. I_{A} V_{1/2} was measured at the end of each drug application and the wash periods. In some cases, the application order was altered as described in Materials and Methods. We found that 10µM flupenthixol reversibly blocked the DA induced shift in LP I_{A} by 73.2± 8.4% (p< 0.001) but had no significant effect on DA induced changes in PD I_{A}. On the other hand, metoclopramide reversibly blocked the DA induced shift in PD I_{A} by 71.6 ± 11.7% (p< 0.001) but had no significant effect on DA induced changes in LP I_{A}.

We next tested the effects of the antagonists on DA induced changes in an ongoing rhythm (Figures 10&11). It is possible to remove all descending input to the STG with a sucrose block (deafferented preparation), and then to test the effect of a given modulator, such as DA, on network output. It was previously shown that under these conditions, DA altered the resting membrane potential and spike frequen-
cy of LP (increased) and PD (decreased) (Flamm and Harris-Warrick 1986). We tested whether our antagonists could block these effects. The STNS was dissected and pinned in a dish; descending input was blocked by applying sucrose to the desheathed stn. Under these conditions, the normally rhythmically bursting LP was silent and rhythmic PD bursting slowed (Figure 10A). A ten minute bath application of $10^{-5}$ M DA significantly depolarized LP on average by $2.0 \pm 0.5$ mV ($p<0.001$) (Figure 10 B&C) and increased spike frequency (Figure 10 D&E). Flupenthixol (Figure 10 B&D) but not metoclopramide (Figure 10 C&E) blocked these effects. DA significantly hyperpolarized PD by $1.9 \pm 0.5$ mV ($p<0.01$) (Figure 10 B&C) and decreased spike frequency by $6.4 \pm 2.6$ Hz ($p<0.05$) (Figure 10 D&E). Metoclopramide (Figure 10 C&E), but not flupenthixol (Figure 10 B&D), prevented these changes. Taken together, the pharmacological experiments confirm that DA alters LP I$_A$ via D1Rs and PD I$_A$ via D2Rs.

D1 and D2 receptors contribute to DA induced changes in cycle frequency in deafferented preparations

In addition to altering the intrinsic properties of individual neurons, DA altered network output in deafferented preparations (Figures 10&11). On average a 10 min, 10$\mu$M DA bath application reduced cycle frequency from $0.78 \pm 0.07$ Hz to $0.48 \pm 0.1$ Hz ($P<0.05$, n=15). However, as can be seen in Figure 11, individual preparations varied such that DA could also increase cycle frequency or have little to no effect in ~27% of the preparations examined. We asked whether D1Rs and/or D2Rs contributed to changes in cycle frequency. In every case, metoclopramide blocked the DA induced change in cycle frequency. On the other hand, the effect of flupenthixol varied from no effect to a complete block of the DA induced change in cycle frequency.

DA appears to have subtle and variable effects on ongoing, spontaneous rhythmic activity

Pyloric output is driven by a conditional pacemaker kernel, such that rhythmic activity is ultimately dependent upon the presence of neuromodulators, such as DA (Bal, Nagy et al. 1988). The STG resides in a blood vessel and is constantly bathed by hemolymph, which may contain nM concentrations of DA (Sullivan, Friend et al. 1977). In addition, monoaminergic systems often use volume neurotransmission, which results in the continuous presence of nM concentrations of monoamines in the extracellular-
lar space (Zoli and Agnati 1996). We have recently shown that DARs in the STG receive volume transmissions from dopaminergic projection neurons (Oginsky, Rodgers et al. 2010). Thus, it is reasonable to think that STG neurons in a combined preparation (no sucrose block on stn) may be continuously exposed to low concentrations of DA with localized, transient increases (up to hundreds of nMs) depending upon projection neuron activity. We used the DAR antagonists to begin to decipher the effect of endogenous DA on spontaneous network activity.

In order to determine D1R and D2R function in combined preparations, prior to and after bath applying 10 µM flupenthixol and/or 10 µM metoclopramide for 10 min, we measured pyloric cycle frequency as well as PD and LP membrane potential and firing parameters, including burst duration, spikes per burst and spike frequency. Figure 12 shows that antagonist effects were subtle and variable. Antagonists applied alone or together could cause an increase, decrease or no change in cycle frequency, and they never altered cycle frequency by more than 9% (Figure 12A). Application of both antagonists affected a larger number of preparations (67%) than application of either metoclopramide (33%) or flupenthixol alone (33%). Antagonist application appeared to have little effect on LP and PD membrane potential (Figure 12B). Receptor blockade never produced more than a 14% change in spike frequency, and often had no effect (Figure 12C). Metoclopramide changed spike frequency more frequently then flupenthixol (33% vs. 8%). Spike frequency (Figure 12C) is the product of burst duration (Figure 12D) and spikes per burst (Figure 12E). The same symbol (e.g. open blue circle) represents the same cell for panels 12C-E within each column. The figure illustrates that changes in spike frequency could be due to changes in burst duration and/or spikes per burst.

Discussion

DA is known to modulate intrinsic neuronal firing properties and synaptic strengths in a number of systems by acting on a plethora of targets in a single cell (Nicola, Surmeier et al. 2000; Surmeier, Ding et al. 2007). DA induced changes in circuit output is less well studied, often because a circuit cannot be isolated and manipulated in its entirety. To better appreciate the basic organizing principles of modulatory
systems, we study a small model circuit, the pyloric network, which can be examined from the cellular to circuit levels.

The differential effects of DA on LP and PD neurons are due to differences in receptor expression

It was previously shown that DA has opposing effects on two identified cell types of the pyloric motor circuit, LP and PD (Flamm and Harris-Warrick 1986; Flamm and Harris-Warrick 1986; Harris-Warrick, Coniglio et al. 1995; Harris-Warrick, Coniglio et al. 1995; Kloppenburg, Levini et al. 1999; Kloppenburg, Zipfel et al. 2000; Johnson, Kloppenburg et al. 2003; Peck, Gaier et al. 2006). DA increases LP excitability by increasing calcium currents ($I_{\text{Ca}}$) and a hyperpolarization activated inward current ($I_h$), while decreasing $I_A$. DA inhibits PD firing by decreasing $I_{\text{Ca}}$ and increasing $I_A$. DA has no effect on PD $I_h$.

Here we demonstrated that the cell specific effects of DA were due to differences in the DA transduction cascade in each cell type. In particular, LP expresses D1Rs and PD expresses D2Rs.

DAR expression was consistent at the protein level for both cell types. Every LP examined expressed somatodendritic $D_{1\alpha\text{Pan}}$ and $D_{1\beta\text{Pan}}$, but not $D_{2\alpha\text{Pan}}$ receptors. Every PD examined expressed D2Rs, but not D1Rs (Oginsky, Rodgers et al. 2010). However, protein levels were not quantified, and receptor number for a given cell type may vary across preparations. Surface receptors in both cell types appeared to be concentrated in synaptic varicosities within the somatodendritic compartment. It is not clear if LP D1Rs are restricted to a subset of synapses, as is the case for PD D2Rs (Oginsky, Rodgers et al. 2010). LP DAR transcript and protein expression were not consistent. Based on protein expression, we would expect to always observe $D_{1\alpha\text{Pan}}$ transcripts and never observe $D_{2\alpha\text{Pan}}$ transcripts in LP cells. Instead, $D_{1\alpha\text{Pan}}$ and $D_{2\alpha\text{Pan}}$ transcripts were observed in ~60-70% of the cells. This discrepancy is most likely due to receptor expression in glial cells, the fact that the number of glial cells isolated with neuronal somata was uncontrolled and low LP D1aPan transcript numbers. This idea is consistent with previous work that suggests that D1Rs and D2Rs are expressed in mammalian and STG glial cells (Bal, Bachelot et al. 1994; Zanassi, Paolillo et al. 1999; Miyazaki, Asanuma et al. 2004; Farber, Pannasch et al. 2005; Oginsky, Rodgers et al. 2010). Variable $D_{2\alpha\text{Pan}}$ transcript levels may also indicate variable $D_{2\alpha\text{Pan}}$ receptor expression in cellular
compartments not examined here, such as the axon (Bucher, Thirumalai et al. 2003) or axon terminal (Clark, Dever et al. 2004).

**D1 transduction cascades operate in LP neurons**

Here, for the first time, we defined a signal transduction cascade operating in an identified pyloric neuron. We showed that DA modulates LP I\(_A\) by acting on D\(_{1\alpha Pan}\) and D\(_{1\beta Pan}\) receptors that couple with a Gs-AC-cAMP-PKA pathway. Interestingly, there is no consensus cAMP-dependent phosphorylation site on shal channels (Baro, Coniglio et al. 1996). Shal channels may contain atypical PKA phosphorylation sites (Shi, Wu et al. 2007). Alternatively PKA may not act directly on the pore-forming subunit of the A-channel (i.e., shal subunits), but rather, target an auxiliary subunit such as KChip (An, Bowlby et al. 2000), or a downstream enzyme (e.g., phosphatase) that then modifies shal subunits.

The fact that DA reduced the peak LP I\(_A\) through D1R induced changes in cAMP may suggest that DA increases the peak I\(_A\) in PD via a D2-Gi/o coupled cascade that decreases cAMP and PKA activity. Indeed, this is the case in D2R expressing striatal MSNs (Perez, White et al. 2006). However, DA may not act simply by reciprocally altering cAMP levels in LP and PD, as DARs can act through non-canonical cascades: D2Rs can signal through PLC\(\beta\) (Hernandez-Lopez, Tkatch et al. 2000; Clark and Baro 2007) and D1Rs can couple with Gq.

**DA elicits consistent changes in I\(_A\), but variable changes in circuit output in deafferented preparations**

We previously showed that bath applied serotonin (5-HT) had a variable effect on circuit output in deafferented preparations, and could increase, decrease or produce no change in cycle frequency (Spitzer, Cymbalyuk et al. 2008). In contrast, 5-HT activation of pyloric 5-HT\(_1\) or 5-HT\(_2\) receptors produced a consistent effect (e.g., increase in a synaptic strength), but the amplitude of the effect varied due to undetermined phenomena (e.g., synaptic plasticity). Our data suggested that since 5-HT had opposing actions on different components of the circuit, the overall effect of 5-HT on circuit output varied accord-
ing to the relative weights of the individual components. This study provides similar findings for the dopaminergic system operating on the pyloric network.

When applied to deafferented preparations, DA produced variable effects on circuit output and could increase, decrease or have no effect on cycle frequency. In contrast, DA consistently altered individual components of the system. In > 90% of the preparations examined, DA decreased the peak LP $I_A$ and increased the peak PD $I_A$ by shifting A-channel activation voltage dependencies positively or negatively, respectively. However, the amplitude of the change in $I_A$ varied, due in part to cell specific differences in the initial baseline $V_{1/2}$, and the absolute value of the shift in the $V_{1/2}$. Other phenomena might also have contributed to the variability, including animal-to-animal variability in ion channel transcript number (Schulz, Goaillard et al. 2007) or D1R number.

**Variable effect of DA on combined preparations**

Previous studies on the pyloric network suggested that modulators constantly regulate ion current densities (Khorkova and Golowasch 2007). Our discovery that the *Panulirus* dopaminergic system employs volume neurotransmission in the STG suggested that nM levels of DA may continuously modulate the pyloric network in a combined preparation (descending neuromodulatory input intact). Here, for the first time, we examined the function of endogenous DA neurotransmission on circuit output.

We first defined specific antagonists for lobster D1Rs (flupenthixol) and D2Rs (metoclopramide) in order to block DA actions. Interestingly, the flupenthixol IC$_{50}$ at D2Rs was $\sim 10^{-7}$M in HEK cells, but $10^{-5}$M flupenthixol had no significant effect on DA induced changes in PD $I_A$ or membrane potential. It seems that the actions of flupenthixol at D2Rs are dependent on the cell type and/or function examined, which is similar to the functional diversity observed for another D2R ligand, aripiprazole (Shapiro, Renock et al. 2003). These types of observations can be explained by the ‘functional selectivity’ hypothesis, which proposes that ligand-receptor interactions are dictated, in part, by the effector system coupled with the receptor (Urban, Clarke et al. 2007). Thus, differences in effector proteins across cell types could change receptor conformation and thereby ligand interactions.
We next applied the antagonists, individually or together, to a combined preparation generating a spontaneous rhythmic pyloric output. The general caveat that the drugs may have unknown effects at other receptors applies. Whereas the data suggested that endogenous levels of DA modulated spontaneous activity in the intact preparation through D1Rs and D2Rs; in all cases examined, the effects of the antagonists were small and variable. The subtle responses may be due to low endogenous concentrations of DA. In addition, DA is one of a number of modulators acting on the STG (Marder and Bucher 2006). It may be that cell and network outputs are stabilized by the actions of all modulators, so that changes in any given modulator alone will have small effects. This is consistent with the fact that the natural variability observed for a given parameter was significantly greater than the change induced by removal of D1Rs and/or D2Rs (e.g., a >2-fold variation in cycle frequency was observed in the population, but DAR antagonists induced a < 10% change in cycle frequency in any given preparation). It is important to note that low extracellular levels of DA may have a greater effect on cellular attributes not examined here. For example, endogenous levels of DA in the striatum appear to be involved in continuously maintaining chromatin structure (Bertran-Gonzalez, Hakansson et al. 2009).

The variable responses to antagonists may stem from a variety of phenomena. Differences in states, synaptic inputs and/or levels of ion channel/receptor expression in LP or PD neurons could vary across preparations, and this could cause preparation-to-preparation variability in cell and circuit responses to the antagonists (Goaillard, Taylor et al. 2009). However, this need not be the case, as the drug-induced changes in LP and PD firing parameters suggested that several antagonist effects were indirect. For example, both flupenthixol and/or metoclopramide application could alter spike frequency in both LP and PD, though each cell type is known to express a single type of DAR that is sensitive to only one of the two drugs.

**Conclusion**

Our present work illuminates the molecular mechanisms underlying DA neuromodulation, which is a prerequisite for understanding normal network function. Moreover, the use of DAR antagonists to
decipher the innate contribution of DA to network output suggests that modulator function varies across individuals and most likely makes only a small contribution to network output, at least under the conditions studied here. It may be that DA plays a more prominent role in regulating circuit characteristics over the long-term. The discrepant localization of DARs and their target channels raises the issue of spatial modulation, which needs to be addressed in the future.

Acknowledgements

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Grants

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References


King DG. Organization of crustacean neuropil. II. Distribution of synaptic contacts on identified motor neurons in lobster stomatogastric ganglion. J Neurocytol 5: 239–266, 1976b.


Table 1. List of drugs tested to screen DAR antagonists

<table>
<thead>
<tr>
<th>Drugs</th>
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<tr>
<td>(-)-Butaclamol</td>
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<td>Clozapine</td>
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<td>Sulpiride</td>
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<td>Haloperidol</td>
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<td>Spiperone HCl</td>
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<td>Methiothepin mesylate</td>
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<td>Metoclopramide</td>
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<td>Cyproheptadine HCl</td>
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<td>N-R(+)-SCH 23390</td>
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<tr>
<td>Chlorpromazine</td>
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<tr>
<td>(+)-Butaclamol</td>
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<tr>
<td>Domperidone</td>
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<tr>
<td>Etioclopride</td>
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<tr>
<td>Fluphenazine 2HCl</td>
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<td>Flupenthixo</td>
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Figure 1: D₁βPan, D₁βPan but not D₂αPan receptors distributed in the LP somatodendritic compartment. Wholemount STG preparations, each containing a single Texas red filled LP, were stained with anti-D₁βPan (A-E), anti-D₁αPan (F-I) or anti-D₂αPan (J-L). n ≥ 5 wholemount preparations for each receptor type. Yellow staining indicates DAR expression in the LP. Green staining represents DAR expression in uni-
dentified cells. Merged confocal projections were made from serial 1 µm confocal optical slices. A. Merged, 3 µm confocal projection from a wholemount preparation showing D_{1βPan} receptor expression in LP perinuclear vesicles (yellow puncta) and unidentified cells (green projections and punta). B. A 3 µm merged confocal projection from the center of the LP soma showing D_{1βPan} receptor expression in the perinuclear vesicles. C. A 4 µm merged confocal projection from deep within the synaptic neuropil showing D_{1βPan} receptor in the cytoplasmic transport vesicles in higher order neurites (arrows). Arrowheads point to putative synaptic varicosities containing D_{1βPan} receptors. D. High magnification 1 µm optical slice showing cytoplasmic transport vesicles in higher order neurite. E. High magnification 3-4 µm projection showing a cluster of LP synaptic terminals, some of which contain D_{1βPan} receptors. The arrow points to the terminal lacking red ring structure. F-H. 4 µm merged confocal projection showing D_{1αPan} receptor expression in the LP soma, primary neurite, higher order neurites and synaptic terminals (pointed by arrows). I. High magnification 1µm optical slice showing the presence of D_{1αPan} receptors on LP synaptic terminals. Arrow points to the terminal lacking red ring structure. J. A 37 µm merged confocal projection showing the absence of detectable D_{2αPan} in the LP soma and primary neurites. Green staining represents the D_{2αPan} from unidentified neurons. K. A 4 µm confocal projection from deep within the synaptic neuropil showing the absence of D_{2αPan} in the higher order neurites and terminals. L. High magnification 1 µm optical slice showing a cluster of LP synaptic terminals lacking D_{2αPan} receptors.
Figure 2: DA induced positive shifts in the voltage dependence of LP $I_A$. A. TEVC recordings under control conditions, with 10 min bath application of $10^{-5}$ M DA and after 30 min washout of DA. Current traces were obtained in response to a series of depolarizing test pulses (from -50 mV to +60 mV in 10 mV increments) following a hyperpolarizing prepulse to -90 mV. All voltage dependent ion channels except $I_A$ were pharmacologically blocked. Scale bars represent 100 ms and 50 nA. B. Conductance-voltage plots for activation (circles) and inactivation (squares) fit with a 1st-order Boltzmann equation. Plots were obtained in the presence (open symbols) and absence (solid symbols) of DA. $n \geq 5$ for each data point. C-D. Preparation-to-preparation variability. Each dot represents the $I_A V_{1/2}$ activation (C) and inactivation (D) for a single individual in the presence (y axis) versus absence (x axis) of 10 µM DA. The line indicates unity and points above and below the line indicate that DA increased or decreased the $V_{1/2}$, respectively.
Figure 3: An AC activator mimicked and, at saturating concentrations, largely occluded the effects of DA on LP $I_A$. $I_A$ was recorded before (baseline) and 10 min after application of 10µM DA, 50µM forskolin, DA+forskolin, or dideoxy-forskolin (dd-forskolin). Every preparation received only one of the four drug treatments. Each drug except dd-forskolin induced a significant and reversible shift in LP $I_A$ $V_{1/2}$ activation (A) and inactivation (B). Asterisks indicate significant difference from DA treatment using a one way ANOVA with a Tukey posthoc test.

Figure 4: The cAMP analog, 8-Br-cAMP, mimicked DA effects on LP $I_A$. $I_A$ was measured before (baseline) and 10 min after application of DA (A) or 8-Br-cAMP (B). Every preparation received only one dose of either drug. The change from baseline is plotted for $I_A$ $V_{1/2}$ activation (squares) inactivation (circles).
Figure 5: The PKA blockers, H-89 and Rp-cAMP, prevented DA induced changes in LP I<sub>A</sub>. I<sub>A</sub> was measured before and after a 10min 10µM DA application and after a 30min wash from DA. Measurements from the first recording served as baseline for the DA and wash comparisons. After the wash, a 10min application of 20µM H89 was followed by a 10minute application of DA+H89. I<sub>A</sub> was measured at the end of each 10 min application. Measurements from the previous wash served as baseline for H89 comparisons. The change from baseline was plotted for V1/2 activation (A) and V1/2 inactivation (B). C-D. The same experiment was performed except that the concentration of DA was 5µM and 1mM Rp-cAMP was substituted for H89. Asterisks indicate significant difference from baseline as determined with paired t-tests.
Figure 6: The PLCβ inhibitor, ET-18-OCH3, did not block DA’s effects on LP $I_A$. $I_A$ was measured before and during a 10min, 10μM DA application, after a 30min washout, and after sequential 10minute applications of 10μM ET-18-OCH3 and ET-18-OCH3+DA. Measurements from the first recording served as baseline for DA and washout comparisons. The washout measurements served as baseline for all subsequent drug applications. Asterisks indicate significant differences from baseline (DA) or washout (DA+ ET-18-OCH3) as determined with a One way ANOVA followed by a Tukey posthoc test.
Figure 7: Dopamine receptor antagonists. cAMP was measured (pmol/mg protein) in cells expressing D1αPan, D1βPan, and D2αPan receptors in the presence of 10⁻⁵ M DA and increasing concentration of flupenthixol or metoclopramide. Data for a given experiment were normalized to the change in cAMP evoked by DA in the absence of antagonist. The change induced by DA + antagonist are expressed as a percent of the change induced by DA alone (indicated as 100%, - - -). Plots represent the average of ≥3 separate experiments and a given point was the average of duplicates within each experiment.
Appendix B.2 Serotonin Transduction Cascades Mediate Variable Changes in Pyloric Network Cycle Frequency in Response to the Same Modulatory Challenge

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Abstract

A fundamental question in systems biology addresses the issue of how flexibility is built into modulatory networks such that they can produce context dependent responses. Here we examine flexibility in the serotonin (5-HT) response system that modulates the cycle frequency ($cf$) of a rhythmic motor output. We found that depending upon the preparation, the same 5min bath application of 5-HT to the pyloric network of the California spiny lobster, \textit{Panulirus interruptus}, could produce a significant increase, decrease or no change in steady state $cf$ relative to baseline. Interestingly, the mean circuit output was not significantly different among preparations prior to 5-HT application. We developed pharmacological tools to examine the preparation-to-preparation variability in the components of the 5-HT response system. We found that the 5-HT response system consisted of at least three separable components: A 5-HT\textsubscript{2βPan}-like component mediated a rapid decrease followed by a sustained increase in $cf$. A 5-HT\textsubscript{1αPan}-like component produced a small and usually gradual increase in $cf$. At least one other component associated with an unknown receptor mediated a sustained decrease in $cf$. The magnitude of the change in $cf$ produced by each component was highly variable, so that when summed they could produce either a net increase, decrease or no change in $cf$ depending upon the preparation. Overall, our research demonstrates that the balance of opposing components of the 5-HT response system determines the direction and magnitude of 5-HT induced change in steady state $cf$ relative to baseline.

\textsuperscript{3} Appendix B.2 was published in Zhang et al. 2008 Journal of Neurophysiology
Keywords: serotonin receptor; Stomatogastric; crustacean; plasticity; pharmacology; neuromodulation

Introduction

Rhythmic motor patterns such as locomotion, respiration and chewing are essential to the function and survival of an animal. The central pattern generators (CPGs) responsible for producing such rhythmic outputs depend on neuromodulation to maintain their cycle and to appropriately modify their output parameters (Thoby-Brisson and Simmers 1998; Marder and Bucher 2001; Fenelon, Le Feuvre et al. 2004; Hooper and DiCaprio 2004; Selverston 2005; Marder and Bucher 2007). It has been shown that the responses of neural circuits to the same neuromodulator can change depending on the experience or physiological state of the animal (Edwards, Heitler et al. 1999; Marder and Bucher 2001). This can occur through changes in synaptic strength, intrinsic neuronal properties, cell morphology and gene expression; however, the signaling cascades leading to these changes, and the way in which these mechanisms are restructured in a behaviorally relevant manner, are not clear (Kandel 2001; Mitchell and Johnson 2003; Carlisle and Kennedy 2005; Davis 2006; Yuan and Chen 2006). The first step in understanding this plasticity in neuromodulatory response systems is to delineate the components of the system and how they vary across preparations in response to the same modulatory input.

The stomatogastric nervous system (STNS) of crustaceans is a well-established model for investigating the circuitry and neuromodulation of motor pattern generation. The STNS contains several small, defined circuits, each of which drives a different set of muscles to produce a patterned activity associated with a specific function. One such circuit, the pyloric network, located in the stomatogastric ganglion (STG), consists of 14 identified neurons that fall into 6 cell types (Selverston, Russell et al. 1976). The intrinsic firing properties and synaptic connectivities of each cell type have been described in detail (Harris-Warrick, Marder et al. 1992; Nusbaum and Beenakker 2002; Selverston 2005). In addition, the effects of neuromodulators, including serotonin (5-HT), have been investigated at the cellular and circuit
levels (Beltz, Eisen et al. 1984; Flamm and Harris-Warrick 1986; Flamm and Harris-Warrick 1986; Katz and Harris-Warrick 1990; Harris-Warrick, Nagy et al. 1992; Harris-Warrick, Johnson et al. 1998; Ayali and Harris-Warrick 1999; Peck, Nakanishi et al. 2001).

In the California spiny lobster, Panulirus interruptus, 5-HT is not found in STG nerve terminals. Instead, it acts solely as a neurohormone to directly alter the firing properties of several pyloric neurons. A ten minute 5-HT application intended to mimic neurohormonal transmission elicited bursting from the anterior burster (AB) neuron (Harris-Warrick and Flamm 1987; Ayali and Harris-Warrick 1999), inhibited firing in the lateral pyloric (LP) and ventricular dilator (VD) neurons, excited the inferior cardiac (IC) neuron, and had no effect on the pyloric dilator (PD) or pyloric constrictor (PY) neurons (Flamm and Harris-Warrick 1986; Ayali and Harris-Warrick 1999). In addition, 5-HT modulated the strength of electrotonic coupling and chemical synapses within the circuit (Johnson and Harris-Warrick 1990; Johnson, Peck et al. 1994; Johnson, Peck et al. 1995). The effects of 5-HT are mediated, at least in part, by cell-specific targeting of ionic currents (Kiehn and Harris-Warrick 1992; Zhang and Harris-Warrick 1995; Harris-Warrick, Johnson et al. 1998; Peck, Nakanishi et al. 2001).

Arthropods are known to express multiple 5-HT receptor (5-HTR) types (Tierney 2001). A total of 5 arthropod 5-HTR subtypes have been cloned and characterized to date: two 5-HT\(_1\) receptors; two 5-HT\(_2\) receptors, and a 5-HT\(_7\) receptor (Witz, Amlaiky et al. 1990; Saudou, Boschert et al. 1992; Colas, Launay et al. 1995; Clark, Dever et al. 2004). In addition, analysis of arthropod genomic databases suggest the presence of three putative monoamine receptors that currently remain uncharacterized (discussed in Clark, Dever et al. 2004; Clark and Baro 2007). The arthropod genome therefore most likely contains from five to eight genes encoding 5-HTRs.

Here we investigate the components of the 5-HT response system that control pyloric cycle frequency (cf) in the California spiny lobster. Application of 5-HT to individual preparations that appeared to be in the same state uncovered latent preparation-to-preparation variability in the network. We found that pyloric networks producing motor outputs with similar \(cfs\) could respond to 5-HT application with either an increase, decrease or no change in pyloric \(cf\), depending upon the preparation. We then identi-
fied pharmacological tools to differentiate the actions of the two previously cloned and characterized crustacean receptors, 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ (Clark et al., 2004; Sosa et al., 2004; Spitzer et al., 2008). Finally, we used these tools to define the components of the 5-HT response system and determine how each component contributes to the variability in a network’s response to the same modulatory input.

Materials and Methods

Animals

Spiny lobsters, *Panulirus interruptus*, were obtained from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained at 16°C in continually filtered and aerated artificial seawater. Animals were fed once per week with raw shrimp. 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 received food.

Chemicals and Cell lines

HEK293 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, fresh amine and agonist stock solutions (10$^{-1}$M) were made 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. To test the effect of DMSO on deafferented preparations, we applied 5-HT for 5 min, washed out for 1 hr, applied DMSO for 5 min and then DMSO+5-HT for 5 min. In the absence of 5-HT, application of DMSO for 5 min did not produce a detectable change in cf (p>0.23, n=4, also see Fig. 5). Neither did DMSO application alter the steady-state 5-HT response. The 5-HT induced change from baseline (average of last ten cycles during 5 min 5-HT application/average for last 10 cycles just before 5-HT
application) was not significantly different in the absence (0.93±0.21) vs. presence of DMSO (0.96±0.24, p>0.71, n=3).

Electrophysiological recordings

Spiny lobsters were anesthetized for at least 30 min on ice after which the STNS was dissected out and pinned in a Sylgard lined Petri dish using standard techniques (Selverston, Russell 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 CaCl₂, 39 Na₂SO₄, 10 MgSO₄, 2 glucose, 4.99 HEPES, 5 TES at pH 7.4. All experiments were carried out at room temperature. Petroleum jelly (Vaseline) wells (1-2 cm 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 2 ml/min with saline or drug solutions and tests with Fast Green prior to every experiment showed that the solution in the STG well was completely exchanged in less than 20 s.

Extracellular recordings from the pyloric dilator nerve (pdn), medial ventricular nerve (mvn) and lateral ventricular nerve (lvn) were obtained with stainless steel pin electrodes and a differential AC amplifier (A-M Systems, Everett, WA) as previously described (Baro, Levini et al. 1997). In some preparations, the activity of the PD neuron was monitored with intracellular somatic recordings using glass microelectrodes filled with 3 M KCl (20-30 MΩ) 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 10 min, the saline solution in the stn well was exchanged for 1 M sucrose to block descending neuromodulatory input. After 1 hr 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 1-(m-chlorophenyl)-piperazine (mCPP), 10 μM 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 another 1hr wash (+)butaclamol was applied for 5min followed by 5min of (+)butaclamol + 5-HT. The preparation was washed for a final 1hr and the experiment was terminated. In some experiments, the order of 5-HT and mCPP application were reversed with no detectable difference in effect. For experiments involving only 5-HT and antagonists (cinanserin or (+)butaclamol), one hour after applying a sucrose block, 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. In several experiments we applied antagonist plus 5-HT before 5-HT only, however, we found that the antagonist effects did not appear to wash out between applications (i.e., both 5-HT applications produced a similar effect, which was not the case when the order was reversed.).

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 DataView 4.7a (W.J. Heitler, University of St. Andrews, Scotland), Microsoft Excel and Matlab (MathWorks, Natick, MA). We measured agonist- and antagonist-induced changes in 2 parameters: pyloric \(cf\) and spikes per burst of the VD (ventricular dilator) neuron. One cycle was defined as the period extending from the first spike in one PD burst to the first spike in the following PD burst. The \(cf\) and number of VD spikes per cycle were first plotted as a function of time for each experiment.

Baseline \(cf\) was determined from 10 cycles just before application of a drug when the preparation was in a periodic state (i.e. 1hr after the sucrose block was applied or at the end of a 1hr wash period). The time course of the 5-HT effect could be divided into three quantifiable phases. As a measure of phase 1 we obtained the nadir, i.e., lowest \(cf\) (averaged for a 10sec window) during the first 50 sec in 5-HT. As a measure of phase 2 we obtained the peak maximal \(cf\) (averaged for a ten sec window) from 50 to 120sec
of 5-HT application. As a measure of phase 3 (i.e. steady-state) we obtained the average \( cf \) during the last minute of 5-HT application.

Difference traces representing the 5-HT\(_{2B}\text{Pan} \) -like component of the 5-HT response system were produced by subtracting \( cf \) plots obtained in the presence of 5-HT and antagonist from those obtained in the presence of 5-HT and absence of antagonist. A script was written in Matlab to perform this function. The data accessed by the script are termed the row data. The result of the analysis is to fit a line to the row data, and this line is termed a trace average. Examples of these analyses are shown in Fig. 6. Each set of left and right panels in Fig. 6 represents the analyses for a single preparation. The left panels show two trace averages plotted on top of their respective row data. Consider a single left panel. The set of blue data points represents the instantaneous \( cf \) recorded during a 5min 5-HT application. These are the data that were processed by the script (i.e., the row data) in order to generate the trace average, which is shown as a blue line. The row data and trace average for the instantaneous \( cf \) obtained in the presence of 5-HT plus antagonist are similarly plotted in green. The trace average for a given set of row data was calculated using uniform 0.15 min time intervals and a sliding window that moved in increments of 0.05 min. Now consider the corresponding right panel. The gold trace was obtained by subtracting the green trace average from the blue trace average. Therefore, the gold difference trace represents the change in \( cf \) mediated by activation of 5-HT\(_{2B}\text{Pan} \) -like receptors in that preparation. Baselines for the trace averages were arbitrarily set to zero prior to subtraction. Thus, at any given point in time, the 5-HT\(_{2B}\text{Pan} \) component is calculated to be: \((f_{5\text{-HT}} - f_{5\text{-HT baseline}}) - (f_{5\text{-HT+antagonist}} - f_{antagonist baseline})\), where 5-HT baseline and antagonist baseline are defined in Fig. 4 and \( f = \) frequency.

Fig. 7A plots the mean of the 24 trace averages obtained in the presence of 5-HT. Figure 7B shows the mean of the 24 trace averages obtained in 5-HT + antagonist. The mean of the 24 difference traces is shown in Fig. 7C.
Statistical analyses were performed with Excel (Microsoft) and Prism (GraphPad) as indicated. The 3-D plots were generated with Sigmaplot (Systat). Unless otherwise indicated, means are followed by the standard error of the mean, and significance was judged to be p<0.05.

**Assay of IP release in cells expressing 5-HT\textsubscript{2βPan}**

5-HT\textsubscript{2βPan} was transiently expressed in HEK293 cell culture, and inositol phosphate (IP) release in cells expressing 5-HT\textsubscript{2βPan} was assayed using previously described protocols (Clark et. al., 2004; Spitzer et al., 2008). Briefly, transiently transfected cells expressing 5-HT\textsubscript{2βPan} were split into wells on a 24-well plate with 1µCi/ml of \textsuperscript{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 inositol phosphates (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 (no drug) wells for every experiment. At least three independent experiments were performed for each drug.

**cAMP concentration determinations in cells expressing 5-HT\textsubscript{1αPan}**

5-HT\textsubscript{1αPan} was stably expressed in an inducible expression system and cyclic AMP levels were determined using a Direct cAMP kit (Assay Designs, Ann Arbor, MI) as previously described (Spitzer et al., 2008). Briefly, 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 adenylyl cyclase, were then added to individual wells and left at 37°C 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 concentration using the Direct cAMP kit and protein concentration using a BCA Protein Assay Kit (Pierce, Rockford, Il). Data are presented as picomoles of cAMP per milligram of protein. At least three independent experiments were performed for each drug.

_Heterologous expression system data analysis_

Data for all pharmacology assays involving the heterologous expression systems were plotted and analyzed in GraphPad Prism v.4. Dose-response curves were fitted with a standard slope top-bottom or bottom-top dose-response curve to calculate EC/IC\textsubscript{50} and efficacy values. Unless otherwise indicated, means are followed by the standard error of the mean.

**Results**

*5-HT application produced a stereotypic inhibition of VD but a variable change in cf*

We began this study by establishing the variable actions of 5-HT on pyloric cf. The pyloric circuit in the STNS of *Panulirus* consists of 6 cell types interconnected by electrical and inhibitory chemical synapses (Fig. 1A). The circuit is driven by a pacemaker ensemble consisting of a single AB pacemaker interneuron that is electrically coupled to two PD motoneurons. In addition, two follower motoneurons, VD (1 per ganglion) and LP (1 per ganglion), feed back onto the pacemaker kernel and play an important role in governing cf (Weaver and Hooper 2003). Endogenous oscillations in the AB neuron and consequent activity of coupled and follower neurons give rise to an identifiable motor output that can be meas-
ured extracellularly on efferent motor nerves. We monitored the AB-PD pacemaker kernel with extracellular recordings from the pdn, a motor nerve that exclusively contains the two axons of the 2 PD neurons. VD was monitored with extracellular electrodes on the mvn, a motor nerve that exclusively contains the axons of the single VD and IC neurons (Fig. 1B, top). Although the LP is also important in governing cf (Miller and Selverston 1982; Beltz, Eisen et al. 1984; Ayali and Harris-Warrick 1999; Weaver and Hooper 2003), and is directly inhibited by 5-HT (Flamm and Harris-Warrick 1986b), LP was silent under our experimental conditions (see below) and not monitored in these studies.

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 stabilizes in a basal cycling state; the pacemaker kernel cycles weakly (Fig. 1B, middle), VD firing is reduced (Fig. 1B, middle) and LP, PY (8 per ganglion) and IC neurons are silenced (not shown). Once endogenous neuromodulation is removed with the sucrose block (deafferented preparation), the effect of individual modulators such as 5-HT can be assessed. We used a 5min application of 5-HT to mimic neurohormonal transmission. This relatively short application was chosen to minimize the induction of long term effects, but still produce a steady state response. The concentration of 5-HT used in these studies (10µM) is likely to be several orders of magnitude higher than the normal hormonal concentration. Thus, the actions of neurohormonal 5-HT in vivo may not be exactly reproduced in this paper. Nevertheless, the use of this high concentration is appropriate here, as the goal of this study was to investigate the latent variability in a network’s response to the same modulatory input.

Consistent with previous reports, we found that 5-HT altered the activity of both the pacemaker kernel and the VD neuron. Fig. 1B shows serial recordings of pdn and mvn activity from two representative preparations before sucrose block (top), 1hr after sucrose block (middle) and at the peak of the 5-HT effect (bottom). As expected, there was a sustained reduction in VD firing frequency throughout the 5min 5-HT application. The VD was inhibited by 100% from baseline in 27 of 28 experiments, and the remaining preparation showed a 95% decrease in firing frequency. On the other hand, a 5min application of 10µM 5-HT did not produce consistent alterations in cf. 5-HT could increase (Fig. 1B, left pdn traces),
decrease (Fig. 1B, right pdn traces) or produce no change (not shown) in cf relative to baseline, where baseline was measured after the 1hr sucrose block, immediately before 10µM 5-HT application.

Fig. 1C emphasizes the variability in 5-HT effects on cf. The average cf for the last 10 cycles in 5-HT (i.e., steady state 5-HT) was plotted against the average for the last 10 cycles just prior to the addition of 5-HT (i.e., baseline) for every deafferented preparation examined (n=44). Each datum represents a single preparation and the standard error of the means. The diagonal line represents unity. Experiments in which 5-HT had no effect fall along the line; those where 5-HT induced increases or decreases in cf are represented by data points above or below the line, respectively. The average steady state 5-HT effect for all 44 preparations is plotted in Fig 1D. The representation of the data in this manner is deceptive as it suggests that 5-HT had no significant effect on pyloric cf, which is clearly not the case (Fig. 1C).

The variable change in cf did not correlate with gender, molt stage, time/date or the presence/absence of a spermatophore. It was previously suggested that 5-HT could elicit an increase in cf when the LP was silent and a decrease when the LP cell was active (Beltz et al., 1984). However, this did not seem to be the case in our hands. We obtained extracellular LP recordings from the lvn in 20 preparations. After descending modulatory input was removed with a sucrose block, the LP was silent in 17 preparations, and fired very weakly and irregularly in the remaining 3. Moreover, it was postulated that the cf decrease was due to LP inhibition of PD. Using 10^{-6}M picrotoxin to suppress glutamatergic transmission (Bidaut 1980), and hence the LP to PD synapse, we still observed an increase or decrease in cf (n=3, data not shown)

Interestingly, washout of 5-HT for 10min produced a significant increase in cf (Fig. 1D). In order to determine whether the washout effect varied with regard to the 5-HT response, we first classified preparations according to their response. Preparations showing more than a 5% decrease in steady state 5-HT cf relative to baseline were called Class S for slower. These preparations showed a mean 48±7% decrease in cf over baseline (paired t-test p<10^{-5}, n=21), with individual decreases ranging from ~12-100%. Preparations falling into Class F, for faster, showed a mean 26±6% increase in steady state 5-HT cf from baseline (paired t-test p=0.001, n=17), and individual increases ranged from ~6-107%. Preparations dis-
playing less than a 5% change were classified as NC, for no change. These preparations showed a mean
0.0±1.0% decrease in cf over baseline (paired t-test p>0.71, n=6), and changes ranged from a 4% decrease
to a 3% increase. The 5-HT effect washed out immediately and completely for all Class S preparations,
and then the effect reversed so that by 1hr of wash the cf (averages for last ten cycles of 1hr wash) had
significantly increased by an average of 26±15% over the original baseline (Fig 2A). Similarly, all Class
F (Fig 2B) and NC (Fig. 2C) preparations showed a significant increase over baseline by a 1hr wash.
Thus it appeared that the washout effect was the same for all Classes of preparations regardless of their
response to 5-HT.

To assess whether the increased baseline was evoked by 5-HT washout or experimental “drift”,
we recorded for the same length of time from 6 deafferented preparations that never received 5-HT. Plots
of instantaneous cycle frequency vs. time showed that in the absence of 5-HT there was a slow steady
increase in cf over the course of 70min (Fig 3A). The average of the last10 cycles during the 1hr sucrose
block represented baseline. Fig 2D illustrates that there was no significant increase in cf over baseline by
5min (i.e. the time corresponding to 5min in 5-HT); however, there was a significant increase over base-
line at the time corresponding to the 1hr wash. These data suggest that 5-HT independent phenomena
(e.g., lack of modulatory inputs) could at least partially underpin the changing baseline over the course of
the experiment.

We were interested in whether the change in baseline cf over 1hr would alter a deafferented prep-
aration’s response to 5-HT. In several experiments (n=8) we applied 5-HT sequentially to a deafferented
preparation: 5-HT was applied for 5min, washed out for 1hr and then reapplied for 5min (Fig 3B). We
observed that the average response to the first and second applications of 5-HT were not significantly dif-
ferent (average steady-state 5-HT/baseline = 0.58±0.15Hz, 1st application vs. 0.54±0.14Hz, 2nd applica-
tion; paired t-test p=0.17, n=8); however, the second response was usually set atop a higher baseline. Im-
portantly, these experiments make the point that 5-HT receptors respond in a like manner to serial 5min
applications of 5-HT spaced at 1hr intervals (see below).

*The three temporal phases of the 5-HT induced changes in cf*
We next examined the time course of the 5-HT induced changes in $cf$ by plotting the frequency of each pyloric cycle before, during and after 5-HT application ($n=37$). Six representative examples are shown in Fig. 3,C-H. In ~75% of the experiments (Fig. 2C-F) the time course could generally be described as having three phases: An immediate decrease within the first 50 sec of 5-HT application (Phase 1), followed by a slow increase over the next 70 sec (Phase 2), which ended in an apparent steady state (Phase 3). The time course and absolute value of the peak within each phase varied according to the preparation. Once peak $cf$ was reached in phase 2, it was either maintained or gradually reached a new apparent steady state (e.g. Fig. 3E vs. 3F). Cycle frequencies were fairly stable after 3 min in 5-HT; however, steady state 5-HT effects varied greatly, such that the three aforementioned Classes of responses were observed (Fig. 3C, Class F; Fig. 3D; Class S; Fig. 3E&F, Class NC). The remaining 25% of the experiments were atypical with regard to time course. These preparations either exhibited no significant response to 5-HT (not shown; Class NC), displayed a small, gradual, significant increase over baseline throughout 5-HT application (Fig. 3G; Class F) or showed a fairly dramatic, significant decrease relative to baseline, followed by an apparent steady state (Fig. 3H; Class S).

Function and Pharmacology of 5-HT$\text{2}\beta\text{Pan}$

We next sought pharmacological tools that would allow us to characterize the roles of the two previously cloned *Panulirus* 5-HT receptors in generating the 5-HT response. The *Panulirus* 5-HT$\text{2}\beta\text{Pan}$ receptor has been shown to respond specifically to 5-HT and to couple to the inositol phosphate (IP) signaling pathway when stably expressed in HEK cells (Clark, Dever et al. 2004). In this study we transiently expressed 5-HT$\text{2}\beta\text{Pan}$ in HEK cells and measured IP release in response to amines and putative pharmacological agents. Non-transfected parental HEK cells did not respond to 5-HT in the IP assay. IP levels showed a dose dependent increase in response to 5-HT in cells expressing 5-HT$\text{2}\beta\text{Pan}$ with an $EC_{50}$ of 52 nM (Fig. 4A, Table 1).

In order to determine a pharmacological profile for 5-HT$\text{2}\beta\text{Pan}$ we tested a suite of putative agonist and antagonist drugs. In an initial overview, all drugs were tested at $10^{-3}$M (agonists) or $10^{-5}$M (antago-
nists). Agonists that produced a significant increase in IP levels in transfected but not parental lines, and antagonists that inhibited the 5-HT induced increase but had no effect on the parental lines, were then used to generate dose response curves. Typical dose response curves for agonists and antagonists are shown in Fig. 4A. From these curves we determined the potency and efficacy of each drug. The EC$_{50}$ or IC$_{50}$ are measures of the potency of a drug and reflect 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 drug efficacies to the maximum 5-HT response, set at 100%. These data are summarized in Tables 1 and 2.

The following agonist potency rank profile was determined for 5-HT$_{2\beta Pan}$: 5-HT > methysergide > 8-OH-DPAT > 2-Me-5-HT > MeOTryp > 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.

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. Putative antagonists had no effect on non-transfected parental HEK cells.

Function and Pharmacology of 5-HT$_{1\alpha Pan}$

We have functionally characterized crustacean 5-HT$_{1\alpha Pan}$ receptors using a heterologous expression system and demonstrated that these receptors negatively couple with cAMP (Spitzer et al., 2008). In this expression system the 5-HT$_{1\alpha Pan}$ gene is stably integrated into the genome of 293-TR-5-HT$_{1\alpha Pan}$ cells, but the cells do not express the receptor until they are chemically induced to do so. Thus, the negative control for this assay is not parental cells, but rather, non-induced 293-TR-5-HT$_{1\alpha Pan}$ cells.
We tested the same suite of potential agonists as above on induced and non-induced 293-TR-5-HT_{1αPan} cells. Fig. 4B shows the effect of increasing concentrations of 5-HT_{1αPan} agonists on cAMP levels in 293-TR-5-HT_{1αPan} cells that were or were not induced to express 5-HT_{1αPan} 18-20hr prior to performing the cAMP assay. Though their potencies (EC_{50}) and efficacies (% 5-HT effect) were different, both 5-HT and mCPP elicited a dose dependent inhibition of forskolin stimulated cAMP accumulation in induced, but not non-induced cells. Some agonists (DOI, 2-Me-5-HT, quipazine) had significant effects on non-induced 293-TR-5-HT_{1αPan} cells, and could therefore not be tested on induced cells (indicated as Bkd in Table 1). For each of the remaining drugs, the EC_{50} and the relative efficacy were calculated from dose response curves similar to those shown in Fig. 4B. In this way we generated an agonist profile for the receptor that is summarized in Table 1. The relative potencies of functional agonists at 5-HT_{1αPan} were: 5-HT > methysergide > α-Me-5-HT > 5-CT > MeOTryp > 8-OH-DPAT > mCPP. 5-HT_{1αPan} did not respond significantly to 10^{-3}M N-acetyl-5-HT.

We also tested antagonists for their ability to inhibit 5-HT_{1αPan} activation by 5-HT (Table 2). The antagonist (10^{-5}M) was applied ten minutes prior to 5-HT and forskolin stimulated cAMP levels were determined. Twenty nine drugs were tested (the suite tested on 5-HT_{2βPan} and eighteen additional drugs). Seven antagonists (clozapine, methiothepin, S(-)propanolol, metergoline, cyproheptadine, SCH23390, S(-)eticlopride) had significant effects on non-induced 5-HT_{1αPan} cells and could therefore not be tested for activity on induced 5-HT_{1αPan} cells (indicated as Bkd in Table 1). None of the remaining 22 drugs was able to significantly block the inhibition of cAMP accumulation resulting from 5-HT activation of 5-HT_{1αPan}, even when low levels (5x10^{-8}M) of 5-HT were used.

Identification of drugs to differentiate 5-HT_{2βPan} and 5-HT_{1αPan}.

Several drugs whose action could differentiate between 5-HT_{2βPan} and 5-HT_{1αPan} were identified (Tables 1 and 2). The agonist mCPP activates 5-HT_{1αPan} but not 5-HT_{2βPan}. Several 5-HT_{2βPan} antagonists that did not block 5-HT_{1αPan} receptors were also identified: (+)butaclamol, cinanserin and ritanserin. We
next used these drugs on the native system to study the role of the 5-HT\textsubscript{2βPan} and 5-HT\textsubscript{1αPan} receptors in modulating pyloric \textit{cf}.

\textit{Blocking the 5-HT\textsubscript{2βPan} transduction cascade significantly alters temporal phases 2 and 3 of the 5-HT response}

Figs. 1-3 illustrate that the 5-HT induced change in \textit{cf} could be divided into 3 temporal phases (Ph1, Ph2, Ph3) and could vary with the preparation (e.g., Classes F, S & NC). The mechanisms underlying this temporal progression and preparation-to-preparation variability were unknown. We next set out to determine whether 5-HT\textsubscript{1αPan} and 5-HT\textsubscript{2βPan} receptors were components of the 5-HT response system that controlled \textit{cf}, and if so, how these components varied with time and across preparations.

We first examined the contribution of 5-HT\textsubscript{2βPan} receptors to 5-HT modulation of \textit{cf}. The antagonists (+)butaclamol and cinanserin block 5-HT activation of 5-HT\textsubscript{2βPan} but not 5-HT\textsubscript{1αPan} receptors (Table 2); therefore, we applied cinanserin or (+)butaclamol with 5-HT (Fig. 5). Unfortunately, cinanserin and (+)butaclamol could not be used on the same preparation because their antagonistic effects did not wash out. This also prevented us from reversing the order of application (i.e., we could not perform 5-HT plus antagonist followed by 5-HT alone). Nevertheless, it was clear from qualitative observations (e.g., compare Figs. 5A vs. 5B) and quantitative measurements (see below) that these drugs produced comparable results. Additionally, preliminary experiments using the 5-HT\textsubscript{2βPan} antagonist, ritanserin (Table 2), yielded similar findings (not shown). The fact that multiple 5-HT\textsubscript{2βPan} antagonists have the same effect increases the likelihood that these experiments are reporting on 5-HT\textsubscript{2βPan} receptor mediated changes in \textit{cf}.

Representative experiments are shown for cinanserin (Fig. 5A, \textit{n}=8) and (+)butaclamol (Fig. 5B, \textit{n}=22). In these serial experiments, modulatory input was blocked with sucrose on the \textit{stn} and the preparation stabilized in a basal cycling state for 1hr. Baseline measurements of \textit{cf} were obtained immediately before a 5min application of 10µM 5-HT (labeled 5-HT baseline in Fig. 5). 5-HT application was followed by a 1hr wash. Since baseline increased and remained steadily elevated throughout these experi-
ments (Figs. 2D&3A), a new baseline (labeled antagonist baseline in Fig. 5) was measured immediately before application of antagonist and represented the last ten cycles of the 1hr wash. Antagonist (10µM) was applied alone for 5min and then together with 10µM 5-HT for 5min followed by a 1hr wash. Antagonists alone had no significant effect on cf (mean cinanserin induced change in cf = -0.5±1.5%, p>0.7, n=8; mean (+)butaclamol induced change in cf = 0.2±1.4%, paired t-test p>0.8, n=22).

Regardless of the network’s initial response to 5-HT (e.g. F, S, NC), in approximately 91% of the experiments 5-HT\textsubscript{2βPan} antagonists consistently altered the 5-HT induced change in cf in two respects. First, the antagonist abolished the rise in cf usually observed during temporal phase 2 of the 5-HT response (see Fig 3C). In the presence of 5-HT alone, cf increased during phase 2 relative to phase 1 in 26 of 30 experiments (average Δcf = 0.17±0.04Hz; paired t-test for Ph2 peak vs. Ph1 nadir p<10\textsuperscript{-3}, n=30). However, in the presence of 5-HT plus antagonist, the phase 2 peak was no longer significantly different from phase 1 nadir (average Δcf (phase 2 peak–phase 1 nadir) = 0.03±0.05Hz; paired t-test for Ph2 peak vs. Ph1 nadir p>0.56, n=30). Second, whereas 5-HT alone produced no significant change in mean steady-state 5-HT cf relative to baseline (see Fig 1D), 5-HT plus antagonist produced a significant decrease in mean steady-state 5-HT cf relative to baseline (mean steady-state cf in 5-HT plus antagonist/baseline = 0.67±0.05, paired t-test p<10\textsuperscript{-7}, n=30). When considered in light of the fact that 5-HT\textsubscript{2βPan} receptors have been localized to the STG neuropil (Clark et al. 2004), these data suggest that 5-HT\textsubscript{2βPan} receptors are part of the 5-HT response system that modulates cf in most preparations. However, because pharmacology across paralogs within a class (e.g. 5-HT\textsubscript{2α} & 5-HT\textsubscript{2β}) is often conserved (Saudou, Boschert et al. 1992), we cannot conclude that the antagonists are acting solely on 5-HT\textsubscript{2βPan} receptors. For this reason we will refer to the component blocked by 5-HT\textsubscript{2βPan} antagonists as the 5-HT\textsubscript{2βPan}-like component.

In Fig. 5, application of antagonist plus 5-HT appeared to restore the preparation to its original, pre-5-HT cf (i.e., 5-HT baseline). Based on this figure one might speculate that the second drug application removed a long-lasting change initiated by the first 5-HT application. Several pieces of evidence
suggest that this is not the case: First, the change in cf elicited by antagonist plus 5-HT did not vary according to whether the drugs were applied directly to a naive preparation (n=5) or followed a prior exposure to 5-HT and a 1hr wash (Fig. 5), and the drug induced change from baseline resulting from these two types of applications were not significantly different (p=0.77). Second, there was no correlation between “5-HT baseline - antagonist baseline” and “antagonist baseline - cf in 5-HT plus antagonist,” (linear regression, p>0.8, n=30). Third, there was no significant correlation between 5-HT baseline and steady state cf in 5-HT plus antagonist (linear regression, p=0.07, n=30).

At least two highly variable components comprise the 5-HT response system

The experiments represented in Fig. 5 suggested that at least two independent components comprised the 5-HT response system that regulated cf: a 5-HT$_{2βPan}$-like component that was lost upon antagonist application and a non-5-HT$_{2βPan}$ component that remained upon antagonist application. We selected 24 of the original 30 antagonist experiments that were relatively periodic throughout the recording interval and computationally isolated their 5-HT$_{2βPan}$-like components. Importantly, we have shown that 5-HT receptors respond in a like manner to serial 5min applications of 5-HT spaced at 1hr intervals (Fig. 3B). Thus, for each experiment we could approximate and visualize the 5-HT$_{2βPan}$-like component of the 5-HT induced change in cf as a difference trace obtained by subtracting the cf plots acquired in the presence vs. absence of antagonist, as described in Materials and Methods. A subset of our 24 analyses that reflects the range of observed variability is shown in Fig. 6. This figure makes the point that the 5-HT induced change in cf (blue trace in left panels, termed blue trace average, see Materials and Methods) is composed of at least two separable and highly distinct components, a 5-HT$_{2βPan}$-like component (gold trace in right panels, termed difference trace, see Materials and Methods) and a non-5-HT$_{2βPan}$ component (green trace in left panels, termed green trace average, see Materials and Methods), which can perhaps be further subdivided (see below). Note that any synergistic effects resulting from the interaction between the components would be lost upon application of the 5-HT$_{2βPan}$ antagonist, and therefore attributed solely to the 5-
HT\textsubscript{2βPan}-like component. Unfortunately, despite a considerable effort, we could not identify specific 5-HT\textsubscript{2βPan} agonists to complement the antagonist studies.

Figure 7 shows the average response for all 24 preparations (Fig. 7A-C) and a quantification of the variability between the 24 preparations (Fig. 7 D-F). The mean of the 24 individual blue trace averages is plotted in Fig. 7A. The three previously described temporal phases of the 5-HT response are demarcated. To quantify the response for an individual preparation, we measured the change from baseline during each of the 3 temporal phases as described in Materials and Methods. The 24 individual preparations are plotted as 24 blue circles in 3 dimensional space in Fig. 7D. Each axis represents one of the three temporal phases. For a given axis (temporal phase), numbers $> 1$ or $< 1$ represent an increase or decrease in $cf$ relative to baseline, respectively. The larger the change, the further the datum is from the number 1. The means and the individual variability for the non-5-HT\textsubscript{2βPan} component (Fig 7B & 7E, respectively) and the 5-HT\textsubscript{2βPan}-like component (Fig. 7C & 7F, respectively) are similarly plotted. There were statistically significant correlations between the amplitudes of the changes in $cf$ relative to baseline during the three temporal phases (e.g. phase 1 $cf$/baseline vs. phase 2 $cf$/baseline). It appeared that the larger the decrease in $cf$ during phase 1, the smaller the increase over baseline in phases 2 and 3 ($p<0.05$, Correlation analysis, Prism). This was true for both individual components as well as the total response.

Whereas the 5-HT\textsubscript{2βPan}-like component was largely responsible for a delayed, sustained increase in $cf$ relative to baseline (Fig. 7C), there was a remarkable amount of preparation-to-preparation variability in this component (Figs. 6&7F). In 2 of the 24 experiments, the 5-HT\textsubscript{2βPan}-like component was comparatively small or absent (e.g., Fig. 6G). In the remaining 22 experiments the difference trace was typically biphasic, such that 5-HT\textsubscript{2βPan}-like receptor activation generally produced an initial decrease in $cf$ during temporal phase 1, followed by a slower increase that peaked during temporal phase 2 and attained a steady state by 5min (i.e., phase 3). In some experiments, however, one or more of these attributes of the 5-HT\textsubscript{2βPan}-like component was absent. For example, the trough in temporal phase 1 was missing in the experiment shown in Fig. 6A, and both the trough and steady state phases were missing in experiment
6D. Despite this variability, the mean nadir in phase 1 and peaks in phases 2 and 3 were significantly different than baseline (mean cf/baseline±stdev=0.76±0.43Hz, 1.51±0.33Hz, and 1.26±0.45Hz; paired t-tests p<0.05; n=24). Note that the calculated mean phase 1 nadir for the 5-HT<sub>2</sub><sup>β</sup> Pan-like component (-0.24) differs from that which is graphed in Fig 7C (>0.1). This is because the exact time of the nadir varied with each preparation (e.g., see Fig 6) so that when all difference traces were averaged, the large nadirs seen in individual preparations were smoothed away in the average and thereby obscured. This is also true to a lesser extent for the phase 2 peak.

The non-5-HT<sub>2</sub><sup>β</sup> Pan component produced an immediate and sustained decrease in cf relative to baseline (Fig. 7B). In some cases the initial decrease in cf could be followed by a small, gradual increase (e.g., Figs. 6C&G). In 3 of the 24 experiments the non-5-HT<sub>2</sub><sup>β</sup> Pan component was absent (e.g., Fig. 6B). In other experiments there was an obvious shift in the time constants of decay between 30 seconds and 2 minutes (e.g., Fig. 6A), and in 6 of 24 experiments there was an obvious increase in cf during this period. Whereas an unknown element may mediate the increase, it is also possible that the 5-HT<sub>2</sub><sup>β</sup> Pan-like component was incompletely blocked by antagonist in these preparations. Though variable, the mean decrease in cf relative to baseline for the non-5-HT<sub>2</sub><sup>β</sup> Pan component was statistically significant during all 3 temporal phases (mean cf/baseline±stdev=0.79±0.19Hz (phase 1), 0.77±0.31Hz (Phase 2) and 0.71±0.22Hz (Phase 3), n=24, paired t-tests p<10<sup>-3</sup>).

It might be questioned whether some or all of the variability in the response to 5-HT results from the response being sensitive to absolute cf immediately before modulator application. For example, in the crab STNS, the crustacean cardioactive peptide (CCAP) hormone consistently increased cycle period during a relatively fast gastric mill rhythm but produced inconsistent results during slower rhythms (Kirby and Nusbaum 2007). Figs. 1C&2 suggest that the steady-state 5-HT effect is not sensitive to baseline cf. We further examined the cycle period dependent effects of 5-HT in all phases of the 5-HT response and for the 2 individual components by performing correlation analyses (Prism, GraphPad) on measurements from these 24 preparations. Baseline frequency vs. the 5-HT induced change in cf [i.e. (cf in 5-
HT)/baseline] for each of the three temporal phases in each of the 24 preparations was plotted. None of the three correlations were significant (p > 0.05). We performed similar analyses for each temporal phase of the non-5-HT$_{2b\text{Pan}}$ component [i.e., antagonist baseline vs. (cf in antagonist+5-HT)/antagonist baseline] and the 5HT$_{2b\text{Pan}}$-like component (baseline vs. difference trace measurements) and found no significant correlations (p > 0.05 in all cases). Thus, the actions of 5-HT appear to be independent of absolute cf in all three temporal phases of the response, even at the level of individual components.

*The sum of the two opposing and variable components determines the Class*

As discussed at the beginning of Results, preparations could be divided into 3 Classes depending upon whether cf increased (F), decreased (S) or remained unchanged (NC) relative to baseline after 5min in 5-HT. Comparisons of the 3 Classes with a one way ANOVA followed by a Tukey posthoc test demonstrated that all Classes were significantly different from each other at steady state (Table 3, last 3 columns, phase 3). Interestingly, one way ANOVAs indicated that there were no significant differences in cycle frequencies between these Classes prior to 5-HT application (p > 0.6 for intact preparations; p > 0.85 for preparations with a sucrose block on the stn, data not shown). These data indicate that circuits that do not appear to be functionally different (e.g., similar cycle frequencies across individuals) can show different responses to the same modulatory challenge.

The existence of the 3 Classes could be due to Class-specific differences in the components of the 5-HT response system (e.g., the 5-HT$_{2b\text{Pan}}$-like component that increases cf is always large in Class F and small in Class S). To address whether or not there were fundamental differences in the 5-HT$_{2b\text{Pan}}$-like component between the three Classes, we used one-way ANOVAs to compare the changes in cf produced by 5-HT$_{2b\text{Pan}}$-like receptor activation during a given temporal phase. Table 3 shows that there were no significant differences in the 5-HT$_{2b\text{Pan}}$-like component between the different Classes for any temporal phase. This can also be seen by examining Fig. 6. For example, the preparations in Figs. 6C and 6F belong to Classes F and S, respectively, and yet they have highly similar 5-HT$_{2b\text{Pan}}$-like components. Comparable analyses indicated that neither were there statistically significant differences in the non-5-HT$_{2b\text{Pan}}$
component between the Classes (Table 3, Fig 6). In sum, the two components displayed preparation-to-preparation variability within a Class; however, when all preparations were considered, the set of components in one Class was no different than in another, and a given component (e.g., producing large vs. small change from baseline) was equally likely to be found in any of the three Classes.

How are different Classes of responses produced from the same set of components? Analyses of individual preparations suggested it was the balance of the two components that determined the Class, such that at steady state $5$-HT$_{2\beta\text{Pan}}^\text{like}>$non-$5$-HT$_{2\beta\text{Pan}}$ for Class F, non-$5$-HT$_{2\beta\text{Pan}}>$5-HT$_{2\beta\text{Pan}}^\text{like}$ for Class S and non-$5$-HT$_{2\beta\text{Pan}}=$5-HT$_{2\beta\text{Pan}}^\text{like}$ for Class NC.

Fig. 6 shows that the 5-HT induced change in $cf$ was the sum of two highly variable components. Initially both components produced a decrease in $cf$. The minima of the two components were not necessarily coincident. Whereas the non-$5$-HT$_{2\beta\text{Pan}}$ component continued to decrease throughout phase 1, the 5-HT$_{2\beta\text{Pan}}^\text{like}$ component reached its nadir and then began to rise late in phase 1. From this point on the two components produced opposing changes in $cf$, and their balance appeared to determine whether 5-HT ultimately produced a net increase, decrease or no change in $cf$ relative to baseline. This is most clearly seen by comparing Figs. 6B, 6E and 6F. One of the largest 5-HT$_{2\beta\text{Pan}}^\text{like}$ components is observed in Fig. 6F (~0.9Hz increase at steady state), but in this experiment 5-HT produced a 32% net decrease in $cf$ at steady-state, because the non-$5$-HT$_{2\beta\text{Pan}}$ component was even larger. The experiment shown in Fig. 6B has the smallest 5-HT$_{2\beta\text{Pan}}^\text{like}$ component (~0.15 Hz increase at steady state), but 5-HT produced a significant 14% net increase at steady state, because the non-$5$-HT$_{2\beta\text{Pan}}$ component was absent. Fig. 6E shows an intermediate 5-HT$_{2\beta\text{Pan}}^\text{like}$ component (~0.4 Hz increase at steady state), yet 5-HT had no net effect on $cf$ in this experiment because the non-$5$-HT$_{2\beta\text{Pan}}$ component was equally large. Thus, the steady state 5-HT response is determined by the sum of opposing components.

This idea that each Class represents a different balance of components is further substantiated by an examination of the means within a Class. In Class F the average 5-HT$_{2\beta\text{Pan}}^\text{like}$ component was approximately 1.6 times larger than the average non-5-HT$_{2\beta\text{Pan}}$ component at steady state (mean % change from
baseline *cf* at steady state produced by each component: 5-HT$_{2\beta Pan}$-like = 44±8% increase, non-5-HT$_{2\beta Pan}$ = 27±8% decrease, *n*=11), but the opposite was true for class S preparations (mean % change from baseline *cf* at steady state produced by each component: 5-HT$_{2\beta Pan}$-like = 8±12% increase, non-5-HT$_{2\beta Pan}$ = 38±9% decrease, *n*=7). Further, using the aforementioned measurements of the 5-HT induced change from baseline during phase 3 (see Fig 7E-F) we obtained a ratio of the components for each preparation at phase 3 (phase 3 5-HT$_{2\beta Pan}$-like component/phase 3 non-5-HT$_{2\beta Pan}$ component). A Kruskal-Wallis followed by a Dunnett’s post-hoc test showed that the ratios were significantly different for Classes F and S (Table 4). Together these analyses suggest that the different Classes represent a change in the balance of opposing components of the 5-HT response system.

*5-HT$_{1\alpha Pan}$-like receptors mediate a slow increase in *cf***

We next examined the role of 5-HT$_{1\alpha Pan}$ receptors in mediating the 5-HT effect on *cf*. 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. While mCPP is a relatively weak agonist of 5-HT$_{1\alpha Pan}$, it was the only one tested that had no activity at 5-HT$_{2\beta Pan}$ in cell culture (Table 1). This criterion was important since 5-HT$_{2\beta Pan}$ activation contributed significantly to 5-HT induced changes in *cf* (Figs. 5-7). In these studies 100µM mCPP was used to activate 5-HT$_{1\alpha Pan}$ receptors, because 10-50µM mCPP was ineffective (Zhang and Harris-Warrick 1994). Given the high concentration of mCPP used, we cannot rule out the action of this drug at other receptors. Thus, the component of the 5-HT response system identified by these experiments will be referred to as the 5-HT$_{1\alpha Pan}$-like component.

Fig. 8A shows an example of a typical mCPP experiment. In this preparation a 5min, 5-HT (10µM) application slowed *cf*. 5-HT application was followed by a 1hr wash and a 5min application of 100µM mCPP. After a further 1hr wash, (+)butaclamol, a 5-HT$_{2\beta Pan}$ antagonist, was applied for 5min followed by butaclamol±mCPP. In some experiments the order of 5-HT and mCPP applications were reversed.
Application of mCPP caused an increase in \( cf \) in all preparations regardless of the 5-HT effect. Figure 8B illustrates that the mean steady state \( cf \), measured as the average during the last minute of mCPP application, significantly increased by 33\( \pm \)4.5\% relative to baseline. The time course of the response varied so that the peak effect was reached between 1-5 min. In 7 of 9 experiments, a steady state was reached within 5 min, and in the remaining 2 preparations the \( cf \) continued to increase throughout the 5 min 5-HT application. The mCPP induced increase was not blocked by (+) butaclamol, and its magnitude varied across preparations by a factor of 5. After a 1 hr wash out of mCPP, there was an increase in \( cf \) relative to baseline, similar to preparations exposed to 5-HT. As shown in Fig 2D&3A, this increase could be due to mCPP independent phenomena.

These data suggest that 5-HT\(_{1a}\)Pan-like receptors could contribute a small, gradual increase in \( cf \) to the non-5-HT\(_{2b}\)Pan component of the 5-HT response system. Unfortunately, the lack of a specific 5-HT\(_{1a}\)Pan antagonist prevented us from testing this hypothesis further. However, if this hypothesis is true, then there must be at least one additional subcomponent that mediates a decrease in \( cf \) and opposes both the increases mediated by 5-HT\(_{1a}\)Pan-like and 5-HT\(_{2b}\)Pan-like receptors at steady state.

5-HT effects on VD could underpin the decrease in \( cf \) associated with the non-5HT\(_{2b}\)Pan component

In the intact system, VD is thought to regulate \( cf \) via its rectifying electrical junctions with the pacemaker kernel. It was previously shown that VD acts to increase \( cf \), most likely by injecting positive current into the AB/PD pacemaker during the depolarizing phase of the pacemaker oscillation (Weaver and Hooper 2003). VD’s governance of \( cf \) is state dependent, such that VD’s influence is greatest at slower cycle frequencies (e.g., \( \leq 1.0 \)), which are typical for the deafferented preparations studied here (Fig. 1D; average baseline \( cf = 0.77\pm0.4\) Hz, \( n=44 \)). In the absence of 5-HT, VD is active in deafferented preparations (Fig. 1B, \( mvn \) trace in middle panels); thus, it is likely that VD provides an accelerating influence on \( cf \) under these experimental conditions. Presumably, phenomena that inhibit VD and/or reduce its electrical coupling with the pacemaker kernel will diminish VD’s accelerating influence on the pacemaker,
and thereby slow \( cf \). It was previously shown that (1) 10\( \mu \)M 5-HT reduces the electrical coupling between VD and the pacemaker kernel by \( \sim 50\% \) (Johnson, Peck et al. 1993); (2) 10\( \mu \)M 5-HT normally hyperpolarizes VD by \(-10\)mV to inhibit firing (Flamm and Harris-Warrick 1986); and (3) all else being equal, a \(-10\)mV hyperpolarization of VD can reduce \( cf \) by 35-86\% (Weaver and Hooper 2003). The typical time course for the 5-HT induced change in VD firing frequency, shown in Fig. 9A, corresponds to that for the non-5HT\(_{2\beta}\)Pan component of the 5-HT response system (e.g. compare green traces in Figs. 6&7 with Fig. 9A). Moreover, none of the 5-HT\(_{2\beta}\)Pan antagonists had any effect on the VD neuron’s response to 5-HT (Figs. 9B-C); and application of mCPP, a 5-HT\(_{1a}\)Pan agonist, also did not alter the firing rate of the VD neuron (Fig. 8C). Thus, neither 5-HT\(_{2\beta}\)Pan-like nor 5-HT\(_{1a}\)Pan-like receptors mediate the 5-HT induced hyperpolarization of VD. Together these data suggest that 5-HT effects on VD could produce the rapid and sustained decrease in \( cf \) associated with the non-5HT\(_{2\beta}\)Pan component of the 5-HT response system.

**5-HT\(_{2\beta}\)Pan-like receptors do not mediate the excitation of IC**

The activity of the IC cell is visible on many mvn recordings. Like LP, IC was silent after the sucrose block was applied. However, consistent with previous studies (Flamm and Harris-Warrick 1986), in some preparations 5-HT elicited spikes in the IC. The 5-HT\(_{2\beta}\)Pan antagonists had no effect on 5-HT induced excitation of IC in any preparation regardless of whether or not 5-HT produced an increase (\( n=8 \), data not shown) or decrease in \( cf \) (\( n=5 \), not shown). Thus, 5-HT\(_{2\beta}\)Pan-like receptors do not mediate the 5-HT induced excitation of IC.

**Discussion**

Most previous studies have focused on the reproducibility of modulatory effects, and conventional wisdom suggests that a given neuroactive substance will elicit a fairly consistent response from a given circuit. However, these teachings are not in keeping with the emerging principles of metamodulation (Katz and Edwards 1999) and plasticity (Nowotny, Szucs et al. 2007). Further, there are several recent examples showing that the effect of a neuroactive substance on a given target can be context dependent.
(Yeh, Musolf et al. 1997; Edwards, Yeh et al. 2002; Mesce 2002; Birmingham, Billimoria et al. 2003; Mitchell and Johnson 2003). In our experiments one function of 5-HT modulation was completely stereotyped (immediate 5-HT induced hyperpolarization of the VD neuron to inhibit spiking). On the other hand, we found that depending upon the preparation, the same 5min, 5-HT application could increase (Class F), decrease (Class S) or have no effect (Class NC) on the frequency of the rhythmic output of the pyloric CPG at steady state. We developed and implemented pharmacological tools to test the hypothesis that the molecular elements mediating 5-HT effects were different between Classes. We discovered that the elements were similar between Classes, and that the 5-HT response system that regulates \( cf \) was composed of at least three separable components. Some of the components produced opposing changes in \( cf \), and each component showed a great deal of preparation-to-preparation variability. Since the effects of the components summed, it was the balance of the components that ultimately determined whether 5-HT produced a net increase, decrease or no change in pyloric \( cf \) at any given point in time.

\[ \text{The three components of the 5-HT response system} \]

Our data suggest that there are at least three distinct components of the 5-HT response system controlling \( cf \). The first component of the 5-HT response system is associated with a 5-HT\(_{2\beta_{Pan}}\)-like transduction cascade(s). This component is typically biphasic and is characterized by an initial decrease followed by a steady state increase in \( cf \). The biphasic response most likely reflects activation of distinct signaling pathways on different time scales. 5-HT\(_{2\beta_{Pan}}\) receptors couple with Gq to activate phospholipase C\( \beta \) and ultimately increase the activity of protein kinase C in HEK cells (Clark, Dever et al. 2004). In mammals, activation of the 5-HT\(_2\) family can additionally stimulate Phospholipase A\(_2\), NO synthesis, and the MAP Kinase cascade (Nebigil, Etienne et al. 2001). It is not clear whether the mechanism giving rise to the bimodal effect we observed involves one or a combination of these (or other) signaling pathways.

Differential localization of the receptor could also contribute to bimodality. Arvanov et al. (Arvanov, Liang et al. 1999) showed that 5-HT\(_{2A}\) receptors generated a biphasic response in rat medial
prefrontal cortex because they were located both pre- and post-synaptically. The subcellular compartmentalization of 5-HT\textsubscript{2βPan} receptors within the synaptic neuropil of the STG is not known. All STG neurons express 5-HT\textsubscript{2βPan}, and this receptor is found throughout the synaptic neuropil, but not in the plasmalemma surrounding the soma or large diameter neurites (Clark, Dever et al. 2004). We previously demonstrated that 5-HT\textsubscript{2βPan} receptors were located on distal axon terminals in PD and PY cells (Clark et al., 2004). Here we have shown that 5-HT\textsubscript{2βPan} receptors do not mediate 5-HT inhibition of VD or excitation of IC; hence, 5-HT\textsubscript{2βPan} receptors may also be localized to the peripheral regions of these neurons. 5-HT is known to regulate multiple conductances in the AB pacemaker neuron that ultimately enhance its excitability and synaptic output to increase steady-state cf (Flamm and Harris-Warrick 1986; Harris-Warrick and Flamm 1987; Johnson, Peck et al. 1993). Furthermore, the 5-HT\textsubscript{2βPan} antagonist, cinanserin, blocks 5-HT elicited AB/PD bursting in the crab, Cancer borealis, (Zhang and Harris-Warrick 1994). The most parsimonious interpretation of all the data is that the 5-HT\textsubscript{2βPan}-like receptors responsible for increasing steady-state cf reside in the AB synaptic neuropil. However, it is also possible that these receptors reside on the terminals of modulatory projection neurons, and that some of the varied effects of 5-HT on cf are mediated by local interactions between these terminals and their targets (Coleman, Nusbaum et al. 1992; Coleman and Nusbaum 1994; Goaillard, Schulz et al. 2004).

The second component of the 5-HT response system is mediated by a 5-HT\textsubscript{1αPan}-like transduction cascade. This component produces a small, gradual increase in cf. 5-HT\textsubscript{1αPan} receptors couple with Gi/o in HEK cells to decrease cAMP (Spitzer et al., 2008). The location of this receptor is presently unknown, though it is unlikely to be found in the VD synaptic neuropil as the 5-HT\textsubscript{1αPan} agonist did not affect VD activity.

The third component of the 5-HT response system results in an immediate and sustained decrease in cf, but the receptors involved are currently unknown. The three uncharacterized GPCRs in the arthropod genome are unlikely candidates because they are not homologous to known 5-HT receptor subtypes (Clark and Baro 2007). We have shown that this component is independent of 5-HT\textsubscript{1} and 5-HT\textsubscript{2}-
like receptors. The only remaining known arthropod 5-HT receptor is 5-HT; which couples with Gs (Schlenstedt, Balfanz et al. 2006).

Our results are consistent with the notion that the third component is at least partially localized to the VD follower neuron. However, this idea is called into question by Ayali and Harris-Warrick (1999), who showed that when modulatory inputs were intact, the 5-HT induced change in cf was not significantly different between the isolated AB neuron, the isolated AB-PD pacemaker kernel, and the intact network. This suggested that 5-HT acted solely on the AB neuron to control cf. Interestingly, in their hands 5-HT always produced an increase in cf. A major difference between the two studies was the presence (Ayali and Harris-Warrick) vs. the absence (this manuscript) of descending modulatory inputs. It is noteworthy that when modulatory inputs to the network are intact (i.e., non-deafferented preparation), LP and VD are both active and have reciprocal effects on cf (Weaver and Hooper, 2003), and that 5-HT acts directly to hyperpolarize both LP and VD to the same extent (Flamm and Harris-Warrick, 1986b). Conversely, in our deafferented preparation, LP is hyperpolarized prior to 5-HT application. Thus, deafferentation may remove a fourth component of the 5-HT response system that resides on the LP and produces an increase in cf.

Sources of component variability

The variability in the three components of the 5-HT response system is striking. Because the pyloric network was isolated from all descending modulatory input in our studies, the variability must be due to relatively stable differences in inherent cellular and/or circuit properties across preparations. Indeed, it has been shown theoretically (Prinz, Bucher et al. 2004) and experimentally (Bucher, Prinz et al. 2005; Schulz, Goaillard et al. 2006) that considerable variability may exist in the cellular and molecular parameters underlying the pyloric network.

The most obvious sources for variability in the response to 5-HT are the proteins comprising the transduction cascades. Receptor function and/or expression can be state-dependent (Ango, Prezeau et al. 2001; Bockaert, Marin et al. 2003; Cirelli and Tononi 2004; Dwivedi, Mondal et al. 2005) (Wohlpart and
Molinoff 1998; Anji, Sullivan Hanley et al. 2001; Riad, Watkins et al. 2001). 5-HT$_{1A}$ expression is highly variable in the crayfish CNS (Spitzer, Antonsen et al. 2005) and preliminary studies suggest this may also be true in the lobster STG (J. Dever, T. Dever, D. Baro, unpublished). On the other hand, overall 5-HT$_{2}$Pan expression appeared to be relatively constant (Clark, Dever et al. 2004), though it was never quantified nor examined in single cells. In addition, one or more downstream components of the 5-HT signal transduction cascades could vary (Kreienkamp 2002; Werry, Sexton et al. 2005). For example, alterations in the signaling pathway could occur at the level of the final target. In this regard, ion channel activity and expression in pyloric neurons is known to vary significantly across individuals (Golowasch, Abbott et al. 1999; Bucher, Prinz et al. 2005; Schulz, Goaillard et al. 2006), and neuromodulatory input can regulate expression of ionic currents and channels over the long-term (Mizrahi, Dickinson et al. 2001; Khorkova and Golowasch 2007).

Synaptic plasticity may also contribute to variability between preparations. 5-HT alters the strengths of glutamatergic synapses between the pacemaker kernel and follower LP and VD neurons, as well as electrical coupling between VD and the pacemaker kernel (Johnson and Harris-Warrick 1990; Johnson, Peck et al. 1994; Johnson, Peck et al. 1995). In preliminary experiments, ablation of glutamatergic synapses did not prevent a variable 5-HT response. To our knowledge, the degree of preparation-to-preparation variability in the strength of electrical coupling between VD and the pacemaker kernel is presently unknown.

State dependent effects that are independent of changes in protein expression and/or morphology could also account for the variability observed here. Many effects of G protein coupled receptors are made manifest only under certain conditions. For example, in prefrontal cortex neurons, activation of dopamine type 1 receptors (D1) significantly reduced EPSP decay time and integral at membrane potentials near spike threshold (i.e., upstate), but not at more hyperpolarized potentials (i.e., downstate). The D1 effect was mediated by changes in the phosphorylation state of Na$^+$ channels (Rotaru, Lewis et al. 2007). D1 activation consistently led to Na$^+$ channel phosphorylation regardless of membrane potential (Carr,
Day et al. 2003); however, phosphorylation resulted in the enhancement of intrinsic slow inactivation, which is a voltage-dependent process that is maximal at depolarized potentials (Carr, Day et al. 2003; Chen, Yu et al. 2006). Thus D1 activation reduced Na\(^+\) channel availability in the upstate, but not in the downstate! Working on STG neurons, Nargeot (2003) demonstrated that the long-term effect of a neuromodulator could be consistent at the molecular level, and yet evoke opposite changes in neuronal activity depending upon the cell’s membrane potential. Similarly, state-dependent effects have been observed at the network level. For example, it has been shown that the influence of the same modulatory change (i.e., change in the strength of an identified synapse) can vary according to network cycle frequency (Thirumalai, Prinz et al. 2006).

In sum, the variability we observed could be due to a variety of mechanisms ranging from changes in gene expression to changes in ion channel phosphorylation states that alter membrane potential. With regard to the latter, it is important to note that the effects of endocrine and paracrine modulation can last well beyond the removal of their inputs (Turrigiano and Selverston 1990; Di Prisco, Pearlstein et al. 1997; Wood, Manor et al. 2004; Blitz, White et al. 2008). This depends, in part, upon the mechanisms for removal of neuroactive substances outside the synapse (Coleman, Konstant et al. 1994; Wood and Nusbaum 2002), as well as the stability of the change induced by the modulator. Since we examined 5-HT actions roughly 2-4 hours after removing hormonal inputs, and only one hour after removing synaptic and paracrine modulatory inputs, their long-term actions could still be in effect.

**Stability vs. variability of neuromodulatory effects on STNS circuits**

In direct contrast to our findings, there exists a vast literature (too large to fully cite here) documenting the reproducibility of neuromodulatory actions on STNS circuits. This apparent discrepancy is easily explained for work examining modulation by projection neurons onto circuit neurons (Saideman, Blitz et al. 2007; Stein, DeLong et al. 2007) and/or extrinsic inputs onto these projection neurons (Beenhakker, Kirby et al. 2007; Blitz, White et al. 2008). Since these types of studies usually employ an intact preparation (i.e., non-deafferented), it may be that reproducibility is maintained through stabilizing
mechanisms involving additional modulatory inputs and/or feedback from STG neurons to input neurons (Coleman and Nusbaum 1994; Coleman, Meyrand et al. 1995; Wood, Manor et al. 2004). The existence of these mechanisms was implied in our studies by the fact that when we removed modulatory input, cycle frequency drifted over time. Additionally, in studies involving projection/sensory neurons, transmission can be highly localized so that only one or a few components of a multi-component modulatory system are activated by a given modulator (e.g., only the excitatory or the inhibitory components). Likewise, the apparent discrepancy is understandable for studies that bath apply neuroactive substances to non-deafferented preparations (Krenz, Nguyen et al. 2000; Szucs, Abarbanel et al. 2005). While the bath-applied modulator would not be localized, the aforementioned stabilizing feedback mechanisms could still enter into picture.

Considering only studies in which modulators were bath applied to deafferented preparations, one observes a large literature reporting consistent effects of modulators on isolated ionic currents in identified neurons (Kloppenburg, Zipfel et al. 2000; Swensen and Marder 2000; Peck, Nakanishi et al. 2001; Peck, Gaier et al. 2006). It must be noted that reproducibility was impossible to validate in these studies, as individual variability was usually obscured in favor of reporting averages. Here we have demonstrated that this may not be the best practice. Nevertheless, assuming the effects were reproducible, their predictability may be explained by the fact that the system was highly reduced. Our work suggests that variability is diminished when examining individual components (e.g. VD inhibition) rather than the interaction of multiple components (e.g., network cf). Studies on these reduced systems suggest that a given transduction cascade produces a similar effect across most preparations. This is in keeping with our findings, which showed that a given component produced comparable effects in all preparations (e.g. increase or decrease), but that it varied with regard to time course and amplitude.

If we consider only those studies that involved bath application to deafferented preparations and that reported on cycle frequency, we find a few reports of variable neuromodulatory effects (Beltz, Eisen et al. 1984; Saideman, Ma et al. 2007); however, the majority of studies suggest that a given neuroactive substance reproducibly alters cycle frequency (Goaillard, Schulz et al. 2004; Perez-Acevedo and Krenz
2005; Christie, Stemmler et al. 2006; Stemmler, Peguero et al. 2007). Again, a lack of individual variability could not be verified, as average responses were usually reported. In many of these cases there was one important difference relative to our study that could account for predictability, and that was that lower concentrations of a neuroactive substance were often employed. Lower concentrations may activate fewer receptor types (i.e. components). For example, the EC$_{50}$ for 5-HT$_{1aPan}$ is nearly an order of magnitude lower than that of 5-HT$_{2bPan}$; thus, at one extreme the 5-HT$_{1aPan}$ component might act alone, whereas at the other extreme both components could be maximally activated.

In sum, the paucity of reports on variable modulatory effects is intriguing. It might suggest that the 5-HT response system is more plastic than most neuromodulatory systems. Alternatively, it may be that variability in neuromodulatory response systems has been highly underestimated, perhaps due to reporting procedures and/or the use of techniques that examine individual components rather than their interactions.

Summary

It is becoming increasingly evident that variability in neuronal modulatory systems and networks is often the rule rather than the exception. In the absence of all other modulatory inputs, a given modulatory challenge will not have consistent actions on a cellular or circuit parameter if that parameter is a target of plasticity mechanisms (reviewed in Marder and Goaillard 2006). It is not known if this variability occurs in vivo, and if so, what its functional significance might be. Presumably the variability could reflect adaptations of the stomatogastric network to changing digestive, life cycle or environmental circumstances. Future investigations on this ideal preparation should provide substantial insight into how multiple modulatory, metamodulatory, and plasticity mechanisms are integrated to maintain an adaptive network.

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Table 1: Agonist profiles of 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ from *Panulirus*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Potency (EC$_{50}$, µM)</th>
<th>Efficacy (% 5-HT activation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT$_{2\beta\text{Pan}}$</td>
<td>5-HT$_{1\alpha\text{Pan}}$</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.052</td>
<td>0.0084</td>
</tr>
<tr>
<td>Dopamine</td>
<td>310</td>
<td>IA</td>
</tr>
<tr>
<td>Octopamine</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>Tyramine</td>
<td>283</td>
<td>IA</td>
</tr>
<tr>
<td>Histamine</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>DOI</td>
<td>4.5</td>
<td>Bkd</td>
</tr>
<tr>
<td>5-CT</td>
<td>6.1</td>
<td>2.2</td>
</tr>
<tr>
<td>2-Me-5-HT</td>
<td>0.78</td>
<td>Bkd</td>
</tr>
<tr>
<td>MeOTryp</td>
<td>1.0</td>
<td>4.2</td>
</tr>
<tr>
<td>N-acetyl-5-HT</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>Quipazine</td>
<td>IA</td>
<td>Bkd</td>
</tr>
<tr>
<td>α-Me-HT</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>0.27</td>
<td>7.6</td>
</tr>
<tr>
<td>mCPP</td>
<td>IA</td>
<td><strong>139</strong></td>
</tr>
<tr>
<td>Methysergide</td>
<td>0.11</td>
<td>0.089</td>
</tr>
</tbody>
</table>

5-HT$_{2\beta\text{Pan}}$ and 5-HT$_{1\alpha\text{Pan}}$ were expressed in cultured HEK cells and tested for second-messenger responses to various pharmacological agents. EC$_{50}$ values (potencies) and relative efficacies 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$_{2\beta\text{Pan}}$ and 5-HT$_{1\alpha\text{Pan}}$ are shown in bold. n $\geq$ 3 repeated experiments for each drug. IA, drug is inactive; Bkd, drug has background activity on the control noninduced or parental cells and was not tested; 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.
Table 2: Antagonist profiles of 5-HT$_{2\beta}$ and 5-HT$_{1\alpha}$ from *Panulirus*.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Potency (IC$_{50}$, µM)</th>
<th>Efficacy (% reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clozapine</td>
<td>6.8</td>
<td>Bkd</td>
</tr>
<tr>
<td>Ritalserin</td>
<td>0.57</td>
<td>IA</td>
</tr>
<tr>
<td>Methiothepin</td>
<td>0.66</td>
<td>Bkd</td>
</tr>
<tr>
<td>(+)-Butaclamol</td>
<td>0.14</td>
<td>IA</td>
</tr>
<tr>
<td>Cinanserin</td>
<td>1.2</td>
<td>IA</td>
</tr>
<tr>
<td>Gramine</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>(-)-Butaclamol</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>Ketanserin</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>Spiperone</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>Prazosin</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>Atropine</td>
<td>IA</td>
<td>IA</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Flupenthixol</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Domperidone</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Fluphenazine</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Metoclopride</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>(-)-Sulpiride</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>WAY100635</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Sc(-)-Propanolol</td>
<td>ND</td>
<td>Bkd</td>
</tr>
<tr>
<td>SB269970</td>
<td>ND</td>
<td>IA</td>
</tr>
<tr>
<td>Metergoline</td>
<td>ND</td>
<td>Bkd</td>
</tr>
<tr>
<td>Cyproheptadine</td>
<td>ND</td>
<td>Bkd</td>
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<tr>
<td>SB224289</td>
<td>ND</td>
<td>IA</td>
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<td>BRL15572</td>
<td>ND</td>
<td>IA</td>
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<tr>
<td>TFMPP</td>
<td>ND</td>
<td>IA</td>
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<tr>
<td>SCH23390</td>
<td>ND</td>
<td>Bkd</td>
</tr>
<tr>
<td>Sc(-)-eticlopride</td>
<td>ND</td>
<td>Bkd</td>
</tr>
</tbody>
</table>

5-HT2βPan and 5-HT1αPan were expressed in cultured HEK cells and tested for second-messenger responses to various pharmacological agents. IC50 values (potencies) and relative efficacies were calculated from dose–response curves for each drug. Efficacy is presented as the percentage reduction of the total effect obtained from 5-HT in the absence of antagonist. Drugs with differential actions on 5-HT2 $\beta$ Pan and 5-HT1 $\alpha$ Pan are shown in bold. n $\geq$ 3 repeated experiments for each drug. IA, inactive; Bkd, drug has background activity on noninduced or parental cells and was not tested; ND, not determined.
### Table 3: Average 5-HT induced fold change relative to baseline in each phase of a given Class

<table>
<thead>
<tr>
<th>Class (n)</th>
<th>5-HT$_{2β}$ Pan–like component (cf/baseline)</th>
<th>non-5-HT$_{2β}$ Pan component (cf in 5-HT+antagonist)/baseline</th>
<th>complete 5-HT response system (cf in 5-HT)/baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (11)</td>
<td>0.86±0.07</td>
<td>0.86±0.04</td>
<td>0.98±0.03</td>
</tr>
<tr>
<td>S (7)</td>
<td>0.74±0.09</td>
<td>0.67±0.08</td>
<td>0.56±0.10</td>
</tr>
<tr>
<td>NC (6)</td>
<td>0.89±0.05</td>
<td>0.72±0.09</td>
<td>0.67±0.06</td>
</tr>
<tr>
<td>phase 1</td>
<td>0.74±0.05</td>
<td>0.67±0.09</td>
<td>0.67±0.06</td>
</tr>
<tr>
<td>F (11)</td>
<td>1.40±0.09</td>
<td>0.81±0.1</td>
<td>1.31±0.06</td>
</tr>
<tr>
<td>S (7)</td>
<td>1.15±0.16</td>
<td>0.65±0.1</td>
<td>0.62±0.18</td>
</tr>
<tr>
<td>NC (6)</td>
<td>1.27±0.07</td>
<td>0.7±0.014</td>
<td>1.05±0.06</td>
</tr>
<tr>
<td>phase 2</td>
<td>0.73±0.07</td>
<td>0.62±0.09</td>
<td>0.67±0.04</td>
</tr>
<tr>
<td>F (11)</td>
<td>1.44±0.08</td>
<td>1.22±0.04</td>
<td>1.05±0.06</td>
</tr>
<tr>
<td>S (7)</td>
<td>1.08±0.12</td>
<td>0.64±0.09</td>
<td>0.98±0.01</td>
</tr>
<tr>
<td>NC (6)</td>
<td>1.4±0.10</td>
<td>1.19±0.18</td>
<td>*</td>
</tr>
<tr>
<td>phase 3</td>
<td>1.44±0.08</td>
<td>1.22±0.04</td>
<td>1.05±0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE for the 5-HT$_{2β}$ Pan–like component, the non-5-HT$_{2β}$ Pan component, and the total response. The 5-HT–induced change from baseline during each temporal phase was calculated as described in METHODS for each of the 24 preparations represented in Figs. 6 and 7. Significant differences between classes were determined for each component or the total response, by comparing preparations using one-way ANOVAs followed by Tukey post hoc tests. We compared data representing only the same temporal phase and the same component (i.e., three adjacent rows). Note that there were no differences in phases 1, 2, or 3 of the 5-HT$_{2β}$ Pan–like component between classes F, S, and NC. Similarly, there were no significant differences in the non-5-HT$_{2β}$ Pan component between classes during temporal phases 1, 2, or 3. However, significant differences between classes could be observed for the total 5-HT response during all three temporal phases.

*†*, significantly different from Class F, P < 0.05;

*‡*, significantly different from Class S, P < 0.05;

*§*, significantly different from Class NC, P < 0.05.

### Table 4: Average component ratios at steady state (Phase 3)

| 5-HT$_{2β}$ Pan–like component (Hz)/ non-5-HT$_{2β}$ Pan component (Hz) |
|--------------------------|--------------------------|--------------------------|
| Class F (11)             | Class S (7)              | Class NC (6)             |
| 3.74±1.75                | 0.55±0.16                | 1.19±0.18                |
Figure A: Neural network diagram.

Figure B: Spike raster plots for 5-HT faster and slower conditions, with intact and sucrose block conditions.

Figure C: Scatter plot showing the relationship between steady-state 5-HT and baseline frequency.

Figure D: Bar graph comparing cycle frequency across Base, 5-HT, and Wash conditions.
FIG. 1. The pyloric rhythm of the stomatogastric nervous system in spiny lobster is differentially modulated by serotonin (5-HT). A: schematic of the pyloric circuit in P. interruptus (based on Ayali and Harris-Warrick 1999). Large unfilled circles represent the 6 pyloric cell types. The number of cells in each cell type is indicated, where lack of a number indicates a unique neuron. Small filled circles represent inhibitory chemical synapses. Diodes and resistors represent electrical coupling. B: sequential recordings from 2 preparations before sucrose block (top), 1 h after sucrose block (middle), and in 10−5 M 5-HT (bottom). Cycle frequency was either increased (left) or decreased by 5-HT (right). For each set of traces, top extracellular recordings were from the pdn, which contains only the 2 pyloric dilator (PD) axons. Thus these traces represent PD activity. Bottom extracellular recordings were from the mvn, which contains the axons of the single inferior cardiac (IC) and the single ventricular dilator (VD) neurons. The IC neuron was silent during these recordings; thus these traces represent VD spiking. C: the effect of 5-HT on pyloric cycle frequency varies with the preparation. The steady-state 5-HT cycle frequency (average for the last 10 cycles during a 5-min 5-HT application) is plotted against the baseline cycle frequency (average from the last 10 cycles during a 1-h sucrose block, just before 5-HT application) for each of 44 preparations. x and y error bars represent the SE. Note that 5-HT usually makes preparations more rhythmic such that the y error bars are significantly smaller than the x (P < 0.002, paired t-test on the 2 sets of SEs). D: on average, 5-HT has no significant effect on pyloric cycle frequency. The bar graphs represent the average baseline and state-state 5-HT response for all 44 preparations plotted in C. Additionally, the average cycle frequency after a 10-min washout of 5-HT is shown (average for the last 10 cycles during a 10-min wash). Error bars represent the SE. A repeated-measures ANOVA was performed followed by a Tukey post hoc test: *, significantly different from baseline (P < 0.05); #, significantly different from steady-state 5-HT (P < 0.05).
FIG. 2. The increase in cycle frequency observed during 10 µM 5-HT washout is at least partially due to 5-HT–independent phenomena. Preparations were classified on the basis of whether the steady-state 5-HT cycle frequency was slower (A), faster (B), or unchanged (C) relative to baseline as indicated in the text. For each Class, the average cycle frequency for baseline, steady-state 5-HT, and 10- and 60-min washes are plotted. D: the same measurements for experiments in which 5-HT was omitted, but that were otherwise identical to those represented in A–C (i.e., mock experiments). Error bars, SE; a repeated-measures ANOVA was performed on each of the 4 data sets (A–D) followed by a Tukey post hoc test: *, significantly different from baseline (P < 0.05); #, significantly different from steady-state 5-HT or steady-state mock 5-HT (P < 0.05).
FIG. 3. The time course of the 5-HT response. A: pyloric cycle frequency slowly increases over the course of 70 min in the absence of modulatory input. Plot of cycle frequency vs. time for a representative mock experiment (n = 6). Each point marks a single pyloric burst. These experiments are exactly like the experiments shown in C–H, except that 5-HT was omitted. B: sequential applications of 5-HT produce similar effects. Representative plot of cycle frequency vs. time for experiments in which a second 5-HT application followed the 1-h wash (n = 8). The gray boxes indicate the times of 5-HT application. C–H: the time course of the 5-HT response is variable and complex. In 37 experiments a sucrose block was placed on the sn for 1 h; 10 µM 5-HT was next applied for 5 min and then washed out for 1 h. Plots of cycle frequency vs. time for 6 representative experiments are shown before, during, and after 5-HT application. One minute of cycles after the 1-h wash is shown after the hash marks in each plot. The vertical lines in C approximate the 3 temporal phases of the response described in the text.
FIG. 4. Signaling and pharmacology of 5-HT2βPan and 5-HT1αPan. A: dose–response curves of inositol phosphate (IP) activation in HEK cells. Nontransfected cells do not respond to 5-HT (crosses). Cells expressing 5-HT2βPan respond to 5-HT and an agonist, 8-OH-DPAT, with EC50 values of 52 and 270 nM, respectively (black squares and circles). An antagonist, cinanserin, blocks 5-HT activation of 5-HT2βPan with an IC50 of 140 nM (gray diamonds). Mean ± SE, n ≥ 3.

B: dose–response curves of cAMP accumulation in 293-TR-5HT1αPan cells. In cells induced to express 5-HT1αPan, forskolin-induced accumulation of cAMP is inhibited in response to 5-HT and an agonist, α-Me-5-HT, with EC50 values of 8.4 nM and 1.1 μM, respectively (black squares and circles). Noninduced cells did not respond significantly to 5-HT (crosses). Mean ± SE, n ≥ 3.
FIG. 5. 5-HT2βPan-like receptors contribute to the 5-HT response. Time-course plots of representative experiments showing pyloric cycle frequency during serial application of 10 μM 5-HT alone and with 10 μM cinanserin, n = 8 (A) or 10 μM (+)butaclamol, n = 22 (B).
FIG. 6. Examples of the computationally isolated 5-HT2βPan-like component of the 5-HT response system. Cycle frequency time-course plots for 7 different preparations are shown (A–G). These experiments are representative of the 24 preparations analyzed. For a given experiment, the frequency plot obtained in 10 µM 5-HT is shown in blue and the frequency plot obtained in 10 µM 5-HT + 10 µM 5-HT2βPan antagonist is shown in green. Each point represents a single cycle and lines indicate the trace averages as described in METHODS. In many cases the trace average is such an accurate fit that it is difficult to distinguish from the raw data. The gold trace on the right is the difference trace obtained by subtracting the response to 5-HT in the presence of antagonist (green trace average) from the 5-HT response (blue trace average) after arbitrarily setting both baselines to zero.
FIG. 7. Average 5-HT–induced changes in cycle frequency are underpinned by significant individual variation in the 5-HT2βPan-like and non-5-HT2βPan components of the 5-HT response system. The same 24 experimental preparations are represented in all panels. The left panels show the mean of the 24 trace averages for preparations treated with 10 μM 5-HT (A) or with 10 μM 5-HT + 10 μM antagonist (B), or the mean of the 24 difference traces (C). The 3 temporal phases of the response are indicated in A. The right panels show the distribution in 3-dimensional space of 5-HT–induced changes in cycle frequency relative to baseline for individual preparations in the absence (D) or presence (E) of antagonist, or for their computationally isolated 5-HT2βPan-like component (F). The 3 dimensions represent the 3 temporal phases of the 5-HT response. The black lines emanating from each circle simply help to portray the exact location of each individual preparation in 2-dimensional space. The colored lines in D–F indicate the number 1 on each axis.
FIG. 8. Cycle frequency may be increased via activation of 5-HT1αPan. Serotonergic inhibition of VD does not occur via 5-HT1αPan. A: application of 100 µM 1-(m-chlorophenyl)-piperazine (mCPP), a 5-HT1αPan agonist, results in an increase of peak cycle frequency regardless of the individual preparation’s response to 5-HT. In this example, 10 µM 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-HT2βPan blocker (+)butaclamol (10 µM, right). B: 100 µM mCPP significantly increases peak cycle frequency. Bar graphs indicate the average percentage change in cycle frequency during sequential application of drugs [(cycle frequency/baseline) × 100]. Error bars indicate the SE. The presence (+) or absence (−) of a given drug is as indicated underneath each bar. Conditions in which both drugs are absent indicate the first and second washouts. The mCPP effect is not changed in the presence of the 5-HT2βPan blocker, (+)butaclamol. C: VD spiking is not changed by mCPP applications. Bar graphs indicate the average percentage change in VD spike frequency during sequential application of drugs [(spike frequency/baseline) × 100]. Error bars indicate the SE. The presence (+) or absence (−) of a given drug is as indicated underneath each bar. Washes are indicated as (−/−). In the case of the first wash, the bar represents the difference between the first and second baselines. Paired t-test, #P < 0.05 vs. baseline.
FIG. 9. 5-HT2βPan is not involved in elimination of VD activity by 5-HT. A: time course graph of a representative experiment showing VD spikes per cycle during serial application of 10 μM 5-HT followed by 10 μM antagonist alone and with 10 μM 5-HT. Serial applications are separated by 1 h of wash. Circled numbers correspond to time points for measurements presented in bar graphs below. Spiking in the VD neuron is completely inhibited in 10 μM 5-HT. This effect is not blocked by either (+)butaclamol (B) or cinanserin (C). Bar graphs in B and C indicate the average percentage change in VD spike frequency during sequential application of drugs [(spike frequency/baseline) × 100]. Note that the baseline is reset between bars 2 and 3. Error bars indicate the SE. The presence (+) or absence (−) of a given drug is as indicated underneath each bar. Washes are indicated as (−/−). In the case of the first wash (circle 2), the bar represents the difference between the first and second baselines (paired t-test, #P < 0.05 vs. baseline).
Appendix C: List of publications


