P90 RIBOSOMAL S6 KINASE 2 (RSK2) DIRECTLY PHOSPHORYLATES THE 5-HT$_{2A}$ SEROTONIN RECEPTOR THEREBY MODULATING SIGNALING

by

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*We also certify that written approval has been obtained for any proprietary material contained therein.
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# LIST OF ABBREVIATIONS

<table>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
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<td>5-HT&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>5-hydroxytryptamine 2A (serotonin-2A) receptor</td>
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<td>5-HTP</td>
<td>5-hydroxytryptophan</td>
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<td>α&lt;sub&gt;B&lt;/sub&gt;AR</td>
<td>alpha 1b adrenergic receptor</td>
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<td>GDP</td>
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<td>immunoblot</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IkB</td>
<td>nuclear factor kappa B inhibitor</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP</td>
<td>inositol phosphate</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>Jak2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>equilibrium inhibition constant</td>
</tr>
</tbody>
</table>
LSD    lysergic acid diethylamide
MAP1   microtubule-associated protein 1
MAPK   mitogen-activated protein kinase
MEF    mouse embryonic fibroblast
MEK/MKK MAPK kinase
MPP-3  MAGUK p55 subfamily member 3
MS     mass spectrometry
mVSMC  mouse vascular smooth muscle cell
MW     molecular weight
NFkB   nuclear factor kappa B
NHE1   Na+/H+ exchanger 1
NMDA   N-methyl-D-aspartate
NTD    N-terminal kinase domain
PAR    protease-activated receptor
PBS    phosphate-buffered saline
PCP    phencyclidine
PDGF   platelet-derived growth factor
PDK1   3-phosphoinositide-dependent protein kinase-1
PDZ    PSD-95/Discs-large/ZO-1 domain
PI3K   phosphatidylinositol 3-kinase
PIP2   phosphatidylinositol-4,5-bisphosphate
PKA    protein kinase A
PKC    protein kinase C
PLA₂ phospholipase A2
PLC phospholipase C
PLD phospholipase D
PTP protein tyrosine phosphatase
PYK2 Ca²⁺ and cell adhesion-regulated FAK family kinase
RAMP receptor activity modifying protein
Rap-1 Ras family GTPase-1
Raf1/MKKK MAPK kinase kinase
RFU relative fluorescence units
RGS regulators of G protein signaling, GTPase activating proteins
RhoA Ras homolog gene family member A
RhoGEF Rho guanine nucleotide exchange factor
RKIP Raf kinase inhibitor protein
ROS reactive oxygen species
RSK p90 ribosomal S6 kinase
RSK2+/+ RSK2 wild type mice
RSK2−/− RSK2 knock-out mice
RTKs receptor tyrosine kinases
SAP97 synapse-associated protein 97
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2 Src homology 2
Sos son-of-sevenless
Src proto oncogene SRC, Rous sarcoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with 0.1% Tween-20</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factor-alpha</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>V2R</td>
<td>V2 vasopressin receptor</td>
</tr>
</tbody>
</table>
Given the pivotal role G protein-coupled receptors (GPCRs) play in physiological responses, various mechanisms have evolved to ensure the tight regulation of GPCR signaling. Classically, agonist exposure leads to an attenuation of receptor responsiveness (i.e. desensitization) through multiple mechanisms including receptor phosphorylation by second messenger-activated protein kinases (e.g. PKA and PKC) and/or GPCR kinases (i.e. GRKs), receptor internalization, and receptor down-regulation. Our laboratory recently discovered that p90 ribosomal S6 kinase 2 (RSK2), a downstream effector of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade, interacts with the 5-hydroxytryptamine 2A (serotonin 2A, 5-HT$_{2A}$) receptor and exerts a “tonic brake” on agonist-mediated receptor signaling. Importantly, genetic deletion of RSK2 potentiates 5-HT$_{2A}$ signaling in fibroblasts without affecting its subcellular distribution, global G protein function, and expression of serotonergic pathway genes. Furthermore, re-introduction of wild-type RSK2—but not ‘kinase-dead’ RSK2 (K451A)—into fibroblasts restores normal 5-HT$_{2A}$ signaling. Therefore it was hypothesized that RSK2 regulates 5-HT$_{2A}$ receptor signaling through direct receptor phosphorylation. In this thesis I significantly advanced our understanding of 5-HT$_{2A}$ receptor regulation by showing that RSK2 directly phosphorylates the 5-HT$_{2A}$ receptor
third intracellular (i3) loop at the conserved residue Ser314, thereby modulating 5-HT2A receptor signaling. I arrived at these conclusions via multiple lines of evidence including \textit{in vitro} kinase experiments, tandem mass spectrometry, and site-directed mutagenesis. These findings were further validated using phospho-specific Western blot analysis, metabolic labeling studies and whole-cell signaling experiments. These results support a novel regulatory mechanism whereby a downstream effector of the ERK/MAPK pathway directly interacts with, phosphorylates, and modulates signaling of the 5-HT2A serotonin receptor. Furthermore I discovered that RSK2 differentially regulates 5-HT2A agonist signaling, thereby leading to altered patterns of functional selectivity. These studies ultimately led to the larger discovery that RSK2 is required for receptor tyrosine kinase (RTK)-mediated desensitization of 5-HT2A receptors. It is attractive to speculate that RTK signaling may be relevant for regulating 5-HT2A receptor signaling \textit{in vivo} given the important role of growth factor signaling in normal physiology. Moreover, this novel regulatory mechanism may have far-reaching implications for human disease in which RTK activity is altered (e.g. neuropsychiatric disorders and cancer).
CHAPTER 1: INTRODUCTION

1.1 The Receptor Concept

Over 100 years of research have established that specific molecules on or in cells (i.e. receptors) have the capacity to bind compounds (i.e. ligands), thereby reading the chemical information contained in these compounds and translating it into a biological response. Considered the foundation for current pharmacological research and pharmaceutical development, ‘the receptor concept’, as it is best known, emerged from early pharmacological studies showing that small amounts of chemicals profoundly affected living systems, and that minor changes to these compounds led to large changes in their activities. Specifically, the pioneering work of John Newport Langley and Paul Ehrlich introduced the concepts of ‘receptive substances’ and ‘chemoreceptors’, respectively (1,2). It was not until Alfred Joseph Clark’s classic work, entitled ‘The Mode of Action of Drugs on Cells’ (3), that it was realized that the biological response to drugs resulted from the unimolecular interaction of a drug with a receptor on the cell surface. In fact, Alfred Clark’s application of chemical laws to drug-receptor action, combined with the concept of drug intrinsic efficacy as proposed by E.J. Ariens and R.P. Stephenson (i.e. different ligands have various capacities to initiate a response) (4,5), formed the basis of modern pharmacodynamic analysis. It follows, then, that ligands can be classified by their ability to induce a full response (i.e. full agonist), zero response (i.e. antagonist or inverse agonist), or a response intermediate between full agonists and antagonists (i.e. partial agonist) (5). It is important to note that the traditional concept of intrinsic efficacy assumes that a ligand’s ability to impart (or reduce) a stimulus once bound to a receptor is an inherent property of the ligand-receptor complex and is system-independent (i.e.
relative order of efficacy is static across all receptor responses). However, a plethora of recent studies at receptor tyrosine kinases and G protein-coupled receptors including neurotensin, dopamine, opioid, cannabinoid, chemokine, beta-adrenergic, vasopressin, somatostatin, and serotonin receptors have shown that ligands exhibit a wide range of efficacies for different receptor behaviors (i.e. relative order of efficacy is dynamic across various receptor responses) (6). This revelation in receptor pharmacology, now known as ‘functional selectivity’, has profound implications for drug discovery and will be discussed in detail in Chapter 5.

1.2 G Protein-Coupled Receptors (GPCRs)

1.2.1 Introduction

G protein-coupled receptors (GPCRs) are heptahelical transmembrane receptors that have evolved to transduce signals from a vast array of ligands in a variety of eukaryotic organisms ranging from unicellular choanoflagellates (7) to yeast, plants, and animals (reviewed in (8)), and are perhaps the best studied receptors in the human genome. Recent sequencing and annotation of the human genome has revealed that nearly 2% of all genes encode GPCRs, with GPCRs comprising more than half (i.e. 64%) of all signal transduction proteins (9). Phylogenetic analysis of the human genome has shown that the GPCR superfamily contains more than 860 family members, which can be further divided into five distinct families: rhodopsin (A), secretin (B), glutamate (C), adhesion, and frizzled/taste2 (10,11). Of these five families, the rhodopsin family (A) is the largest, with an estimated 752 receptors, of which 258 are non-olfactory (12).
GPCRs mediate nearly every physiological process in the human body ranging from the perception of light and taste to insulin secretion and platelet aggregation by binding and transducing signals from a bewildering array of endogenous ligands (e.g. biogenic and traceamines, peptides, amino acids, glycoproteins, prostanoids, phospholipids, fatty acids, nucleosides, nucleotides, Ca\(^{2+}\) ions) and exogenous ligands (e.g. odorants, bitter and sweet tastants, pheromones, and photons of light) (13). Accordingly, given their central role in cellular signaling, GPCR dysregulation is associated with many disease states. These include retinitis pigmentosa which is caused by constitutively active mutants of rhodopsin, hyperthyroidism which results from mutations in the thyroid stimulating hormone receptor, male precocious puberty which results from constitutively active mutants of the luteinizing hormone receptor, nephrogenic diabetes insipidus which is caused by mutations in the V\(_2\)R, and many cardiovascular disorders which are associated with mutations in the AT\(_1\)R (reviewed in (14)). GPCR mutations are also associated with altered pharmacological responses to drugs (15), a finding that has given rise to research associating genetic differences with altered drug responses (i.e. pharmacogenetics). Taken together, these findings highlight the importance of targeting GPCRs, not only to develop potential therapeutics, but to gain a deeper understanding of GPCR function.

1.2.2 GPCR Structure

Early cloning and elucidation of the primary sequence of bovine rhodopsin (16), combined with hydrophobicity analysis (17,18), suggested that rhodopsin is comprised of seven transmembrane helices (Figure 1-1A). In fact, projection maps derived from cryo-
electron crystallography of 2-D rhodopsin crystals suggested that bovine rhodopsin exists as a bundle of seven transmembrane helices oriented perpendicular to the membrane (Figure 1-1B and C) (19,20). Interestingly, the helical orientation of bovine rhodopsin, as determined from structural information derived from the sequence of 204 GPCRs and the 2-D projection map of bovine rhodopsin, differs significantly from that of the heptahelical light-sensing proton pump bacteriorhodopsin most likely due to the latter’s function as a proton pump and inability to couple to G proteins (Figure 1-1D) (21). Cryo-electron crystallography of 2-D frog rhodopsin crystals confirmed the helical assignments of previous projection maps of rhodopsin (Figure 1-1E) (22). However, with the advent of cryo-crystallography, the availability of synchrotron X-rays, and the growth of new crystals, the first high resolution (2.8Å) crystal structure of a GPCR, bovine rhodopsin, was solved (Figure 1-1F) (23). In agreement with previous low resolution structures, the crystal structure of bovine rhodopsin revealed a highly organized heptahelical transmembrane bundle connected by three extracellular (e1-3) and three intracellular (i1-3) loops, and an extracellular N-terminus and intracellular C-terminus. Interestingly, an eighth helix was identified immediately after TM7 and contained the highly conserved N-P-X-X-Y motif that is reported to be important for interactions with various proteins. Not surprisingly, the helical arrangement in rhodopsin differed markedly from bacteriorhodopsin. In the eight years following that discovery, 15 bovine rhodopsin (or opsins) crystal structures have been published, including the elusive photo-activated rhodopsin and opsins bound to a transducin peptide (24). In addition, two squid rhodopsin structures that couple to the G protein Gaq have been published that exhibit a uniquely ordered i3 loop (25).
Considering the intermediate size of rhodopsin (i.e. 348 amino acids) and the high resolution assignment of secondary structural elements including the position of highly conserved amino acids, the crystal structure of rhodopsin has provided insight into the function of other GPCRs. Most importantly, this has allowed the construction and validation of homology models of non-opsin GPCRs which have been pivotal for discoveries in virtually every aspect of GPCR research ranging from ligand discovery and optimization to elucidation of GPCR conformational states (26,27). However, it is now realized that rhodopsin, a highly specialized GPCR, may not be the ideal representative upon which other GPCRs are based. Fortunately, the past few years have witnessed an explosion in non-opsin GPCR crystal structures including the much anticipated beta-adrenergic receptors (β₁AR and β₂AR) and the adenosine 2A (A₂A) receptor (28-32) reviewed in (33). It follows that these new crystal structures will allow for the development of alternative templates for the creation of new homology models. It is interesting to note that while crystal structures exist for 1) GPCRs, 2) the G protein α subunit, 3) the G protein αβγ heterotrimer, 4) a G protein/RGS complex, 5) β-arrestin, 6) GPCR kinase (GRK), 7) a GRK/G protein complex, and 8) opsin/G protein peptide complex (33), scientists have yet to crystallize a GPCR complexed with an interacting protein (e.g. G protein, arrestin, or GRK). This is an intense area of study and elucidation of such a structure will profoundly alter the trajectory of GPCR research.
**Figure 1-1. The progressive elucidation of a high resolution GPCR crystal structure.**

A, The complete bovine opsin gene was determined via the isolation and identification of bovine opsin cDNA clones followed by the use of these clones to
isolate cloned genomic DNA segments. Nucleotide sequence analysis of genomic DNA provided a complete amino acid sequence which, when coupled with hydrophobicity analysis (schematic on left), allowed preliminary determination of seven 21 amino acid transmembrane segments (TM, represented by lines on left, schematic on right). Adapted from (16). B, Projection density map of bovine rhodopsin at 9.0Å determined from cryo-electron crystallography of 2-D bovine rhodopsin crystals. This panel shows a cartoon rendering of the merged image amplitudes and phases obtained from 13 independent crystalline areas. Adapted from (20). C, The projection density map of a single bovine rhodopsin molecule shows an elongated arc-shaped feature (i.e. three tilted helices) and four resolved peaks of intensity (four transmembrane helices oriented perpendicular to the membrane). Adapted from (20). D, Diagram showing the orientation of the predicted seven transmembrane helices of bovine rhodopsin, as determined from structural information derived from the sequence of 204 GPCRs and the 2-D projection map of bovine rhodopsin. The helical orientation differs markedly from that of the heptahelical light-sensing proton pump bacteriorhodopsin. Adapted from (21). E, Cartoon rendering of density peaks from a slice taken from the 3-D model of frog rhodopsin (cryo-electron crystallography of 2-D crystals; 12Å from the cytoplasmic side of rhodopsin) confirmed the helical assignments of previous projection maps. Adapted from (22). F, High resolution crystal structure of a GPCR (shown here is 5-HT_{2A} homology model) determined from X-ray crystallographic analysis of rhodopsin (2.8Å) crystallized from mixed micelles (23).
1.2.3 Mechanism of GPCR Activation

The mechanism(s) whereby ligands activate GPCRs remain largely unknown despite recent crystallographic efforts including photo-activated rhodopsin and opsin bound to a transducin peptide (24). However, functional experiments and low-resolution biophysical studies have provided insight into receptor activation. It is known that proteins are structurally dynamic and undergo rapid, small scale structural fluctuations (34), thus GPCRs are thought to exist in a continuum of conformations ranging from inactive to maximally active. In fact, studies with the β2AR show that agonists bind to and activate GPCRs through a series of conformational intermediates, and transition to these intermediate states involves disruption of non-covalent interactions that stabilize the basal state (27). Several mechanisms have been proposed to explain receptor activation (i.e. ‘molecular switches’) including 1) a conserved salt bridge between D/E-R-Y in TM3 and an acidic residue in TM6 (i.e. ‘ionic lock’) (35,36), 2) the ‘rotomer toggle switch’ which involves a bend in TM6 around the conserved Pro6.50 (where appropriate, amino acids have been labeled by a numbering scheme introduced in 1995 by Ballesteros and Weinstein (37) which refers to the amino acid relative to a conserved reference amino acid) (38), and 3) the ‘global toggle switch model’ which suggests opposing outward and inward rigid body movements of intracellular and extracellular transmembrane segments, respectively (39). Recent developments including non-rhodopsin crystal structures and additional complementary biophysical techniques (e.g. azido probes (40)) have yielded insight into receptor activation and will be extremely important for furthering our understanding of GPCR activation.
1.2.4 GPCR Signal Transduction: Primary Signaling Pathways

Once GPCRs adopt an active conformation, either through ligand-dependent or - independent processes (i.e. constitutive activity), they function as large guanine nucleotide exchange factors (GEFs) to catalyze the exchange of GDP (inactive) for GTP (active) on the Ga subunit of heterotrimeric G proteins (Figure 1-2). Subsequent to GTP loading, the G protein heterotrimer dissociates into Ga\(_x\)-GTP (where ‘x’ denotes s, i/o, q/11 and 12/13 subclasses) and G\(\beta\gamma\) signaling subunits. To date 28\(\alpha\)-, 5\(\beta\)-, and 12\(\gamma\)-subunits have been identified, thereby allowing for increased specificity and complexity of signaling (13). These two subunits, once liberated from each other, modulate the activity of various effector proteins such as adenylyl cyclases (Ga\(_s\) activates, whereas Ga\(_i\) and G\(\beta\gamma\) inhibit), phospholipase C \(\beta\) (PLC\(\beta\)) isoforms (both Gaq/11 and G\(\beta\gamma\) activate), potassium channels (G\(\beta\gamma\) activate), calcium channels (G\(\beta\gamma\) inhibit), Rho guanine nucleotide exchange factors (RhoGEFs; Ga12/13 activate), among others (reviewed in (13,41)) (Figure1-2). For some effectors, activation leads to the production of small molecule ‘second messengers’ (i.e. cyclic AMP (cAMP), inositol phosphate (IP), diacylglycerol (DAG), and Ca\(^{2+}\)) that activate downstream proteins (e.g. protein kinase A (PKA), protein kinase C (PKC), and calmodulin (CaM)) which, in turn, modulate the activity of GPCRs and other proteins. In addition to mechanisms described below, GPCR signal transduction is attenuated by the intrinsic GTP hydrolysis activity (i.e. GTPase activity) of the Ga subunit, alone or with the aid of recently identified GTPase accelerating proteins (i.e. GAPs, also known as ‘regulators of G protein signaling’ or ‘RGS’ proteins) (42). Thus, GTP hydrolysis functions to both inhibit G protein-mediated
signal transduction by reconstituting the G protein heterotrimer and reset the activation cycle (Figure 1-2).

Figure 1-2. Primary signal transduction pathways of GPCRs. Upon the adoption of an activated receptor conformation, GPCRs function as large guanine nucleotide exchange factors (GEFs) to catalyze the exchange of GDP (inactive) for GTP (active) on the Gα subunit of heterotrimeric G proteins. Once GTP-bound, the Gα subunit dissociates from the undissociable Gβγ heterodimer thereby initiating a plethora of downstream signaling events. Signal transduction is subsequently terminated via hydrolysis of the Gα-bound GTP thereby reconstituting the Gαβγ heterotrimer and effectively sequestering both Gα and Gβγ signaling subunits. This occurs either
through the intrinsic GTP hydrolysis activity (i.e. GTPase activity) of Ga or the GTPase accelerating activity of regulators of G protein signaling (RGS) proteins.

It is important to note that the activation process extending from ligand binding to second messenger production and ultimately to cellular response is not a random event but is exquisitely ordered. In fact, considerable cross-talk has been shown to exist at the level of effector activation and second-messenger production (reviewed in (43)). For instance, activated PKC, in addition to its inhibitory actions by phosphorylating GPCRs, has been shown to increase adenylyl cyclase activation by Gαs. Moreover, Gαq-mediated Ca\(^{2+}\) release is known to both activate and inhibit Ca\(^{2+}\) sensitive adenylyl cyclase isoforms (44). It has also been shown that GPCRs interact with other proteins, whereby GPCRs are incorporated into ‘signalsomes’ that modify GPCR pharmacology and trafficking, limit signaling to predetermined pathways, and position downstream effectors for efficient signaling (45). These interactions include the formation of GPCR dimers (46,47), interactions with receptor activity-modifying proteins (RAMPs) (48), and the binding of PDZ and non-PDZ domain scaffolds (49-51).

1.2.5 GPCR Activation and Signal Transduction: Secondary Pathways Involving Arrestins and GRKs

It is apparent that G protein-mediated signaling accounts for the majority of GPCR action. However, recent reports suggest that GPCRs initiate G protein-independent signal transduction cascades by recruiting proteins previously shown to inhibit classical G protein-mediated signaling (Figure1-3). These include members of the arrestin and GRK protein families. Originally discovered in HEK293 cells, Luttrell et al. (52) showed
that β-arrestins bind directly to Src-family non-receptor tyrosine kinases and recruit them to βARs, thereby promoting extracellular signal-regulated kinase 1/2 (ERK1/2) activation. Since then various additional potential mediators for arrestin-dependent signaling have been identified (e.g. the Ask1, MKK4, and JNK3 signaling module) and associated with an equally diverse set of responses including chemotaxis and anti-apoptosis (reviewed in (45,53)). Significantly, arrestin-dependent ERK1/2 activation has been shown for some G protein-uncoupled mutant receptors and inverse agonists, thereby suggesting that arrestin-dependent signaling can occur independently of heterotrimeric G protein activation (54,55). Thus it can be imagined that GPCRs function as ligand-regulated arrestin docking sites since recruitment of arrestin to the plasma membrane is sufficient to activate ERK1/2 (56). Moreover, the ability for ligands to ‘select’ for distinct GPCR-mediated functions such as ‘arrestinergic’ signaling (i.e. functional selectivity, discussed in Chapter 5) provides the opportunity to develop pathway-specific ligands with valuable therapeutic properties. For instance, the βAR inverse agonist carvedilol, which is effective in the treatment of heart failure, fails to activate Gαs but stimulates activation of ERK1/2 via arrestin2 (57). Therefore ‘arrestinergic’ signaling may contribute to the special efficacy of carvedilol in the treatment of heart failure and may serve as a prototype for a new generation of therapeutic βAR ligands.

Similar to arrestins, several studies have identified instances wherein GRKs promote signaling. For instance, GRK5 and GRK6 are required for arrestin-dependent ERK activation by angiotensin II type 1 receptor (AT1AR), V2 vasopressin receptor (V2R), and β2AR; whereas on the other hand GRK2 and GRK3 are known to inhibit ERK activation. Thus it appears that the GRK5/6 and GRK2/3 sub-families compete with each
other for ERK activation, most likely to promote a balance between desensitization and signaling as well as coordinate the functions of the two pathways (53). Additional data suggest that GRKs might modulate cellular functions in a phosphorylation-independent as a result of their ability to interact with signaling and trafficking proteins including Gαq, Gβγ, PI3K, clathrin, and caveolin (58). Moreover, novel interactions with Raf kinase inhibitor protein (RKIP), Akt, and MEK lead to inhibition of their respective cellular functions (53) (Figure 1-3).

Figure 1-3. Secondary signal transduction pathways of GPCRs involving arrestins and GPCR kinases (GRKs). GPCRs initiate G protein-independent signal transduction cascades by recruiting proteins previously shown to inhibit classical G
protein-mediated signaling. As shown here, GPCR activation leads to the recruitment of both arrestins and GRKs which either activate (+) or inhibit (-) numerous downstream signaling molecules. ‘Arrestinergic’ signaling is important for chemotaxis, anti-apoptosis, and the expression of various genes. GRKs, on the other hand, can modulate cellular functions by both phosphorylation-dependent (e.g. GRK5/6 activation of ERK) and –independent (interaction with RKIP) mechanisms. Abbreviations are: c-Src, proto oncogene SRC, Rous sarcoma; Hck, non-receptor tyrosine protein kinase Hck; Fgr, Gardner Rasheed feline sarcoma viral oncogene homolog; Yes, non-receptor protein tyrosine kinase Yes; ERK, extracellular signal regulated kinase; p38, p38 mitogen activated protein kinase; JNK, cJun N-terminal kinase; RhoA, Ras homolog gene family member A; PP2A, protein phosphatase 2A; Akt, protein kinase B; NFkB, nuclear factor kappa B, IkB, nuclear factor kappa B inhibitor; MEK1, MAPK kinase 1; RKIP, Raf kinase inhibitor protein; Raf1, MAPK kinase kinase 1; PI3K, phosphoinositide 3 kinase. This figure was adapted from (53).

1.2.6 GPCR Activation and Signal Transduction: Secondary Pathways Involving Transactivation of the ERK/MAPK Cascade

Protein phosphorylation, in general, is an indispensable post-translational regulatory mechanism mediated by protein kinases and exploited by the cell to modulate protein signaling cascades, enzyme catalysis, and protein-protein interactions (59). The utility of protein phosphorylation is perhaps best exemplified by the mitogen-activated protein kinases (MAPKs). MAPKs, whose function and regulation are evolutionarily
conserved, phosphorylate specific serines and threonines of target proteins (e.g. other kinases, phospholipases, transcription factors, and cytoskeletal proteins) as part of a ‘phosphorelay system’ to regulate cellular activities including gene expression, mitosis, movement, metabolism, and programmed cell death (i.e. apoptosis) (60). The three main subfamilies of MAPKs, each of which are activated by an assortment of upstream kinases (termed MAPK kinase (MKK) and MAPK kinase kinase (MKKK)), include 1) ERK 1 and 2; 2) c-Jun N-terminal kinases (JNK) 1, 2, and 3; and 3) p38 enzymes p38α, β, γ, and δ (60). ERK1 and 2 are widely expressed and regulate meiosis, mitosis, and post-mitotic functions in differentiated cells (e.g. learning and memory in the CNS, (61)) and are activated by the proto-oncogene Ras in response to growth factors, cytokines, GPCR ligands, viral infection, transforming agents, and carcinogens. As such, the ERK pathway is a popular target for anti-cancer drugs. The JNK subfamily of MAPKs is activated downstream of a large set of MKKKs (i.e. at least 13) which respond to a variety of signals elicited by environmental stresses, growth factors, and radiation and are important for controlling apoptosis (62). Like the JNKs, p38 MAPKs control the expression of many cytokines and are activated by stresses including osmotic and heat shock, inflammatory cytokines, and growth factors (63).

In particular, MAPKs are frequently activated by a large family of cell surface receptors with intrinsic protein tyrosine kinase activity (i.e. receptor tyrosine kinases, or RTKs). Analysis of the human genome has identified 58 RTKs, which are grouped into 20 subfamilies based on kinase domain sequence (64). These RTKs play an important role in controlling some of the most fundamental processes including cell cycle, cell migration, cell metabolism and survival, and cell proliferation and differentiation (65);
however, their abnormal activation is associated with a variety of human cancers (66). RTKs are composed of an extracellular ligand binding domain, a single transmembrane helix, and a C-terminal domain that harbors a conserved protein tyrosine kinase core and regulatory sequences that are subject to phosphorylation by other RTKs or heterologous kinases (e.g. PKC) (Figure 1-4). Recent crystal structures of EGF receptor family RTKs have provided insight into ligand binding and kinase activation (67), suggesting that most RTKs exist as monomers in the membrane (i.e. except for insulin receptors), and upon activation homo- or heterodimerize to facilitate tyrosine phosphorylation across receptor dimers (Figure 1-4). As a result, tyrosine phosphorylation promotes the recruitment of a vast array of downstream effectors that contain Src homology 2 (SH2) or phospho-tyrosine binding (PTB) domains. For the EGF receptor family of RTKs, the assortment of phospho-tyrosine binding proteins provides richness to RTK signal transduction, and when combined with the variety of dimers, contributes to the specificity and potency of EGF receptor signaling (66).

As early as 1980 (68) GPCR ligands (e.g. vasopressin and thrombin) were identified as potent cellular growth factors (69). However, it appeared that this mitogenic response to GPCR ligands did not follow classical G protein signaling models requiring activation of the second messenger-dependent protein kinases PKA and PKC (70). It was subsequently determined that both GPCRs and receptor tyrosine kinases (RTKs), once activated, converged upon tyrosine phosphorylation of Shc and Ras (71-74). At first perplexing, GPCR-mediated activation of a pathway classically assigned to RTK signaling was reconciled with studies showing that GPCR activation promotes phosphorylation of RTKs such as the EGF receptor (i.e. ‘transactivation’) (75-77). In this
way, GPCRs such as thrombin, angiotensin, endothelin, acetylcholine, and lysophosphatidic acid (LPA) receptors, among others, are thought to ‘hijack’ the ERK/MAPK cascade through RTK transactivation (78,79).

Unlike RTK- or non-receptor tyrosine kinase-mediated activation of the ERK/MAPK signaling cascade, the events connecting GPCR activation with RTK phosphorylation or ‘transactivation’ are not well-known and continue to be the subject of intense investigation. The best understood mechanism of transactivation results from the activation of membrane metalloproteases of the ADAM (cell surface enzymes that contain a disintegrin and metalloprotease domain) family and subsequent autocrine/paracrine release of EGF receptor ligands such as heparin-binding epidermal growth factor (HB-EGF) from the cell surface (i.e. ‘ectodomain shedding’) (80,81). Both Gαq/11 and Gβγ (after dissociation from Gai) subunits activate members of the ADAM family of matrix metalloproteases, perhaps via SH3 domain recruitment of activated Src kinase (82), thus resulting in the recruitment and scaffolding of a Ras activation complex and classical growth factor-mediated activation of the ERK/MAPK cascade (Figure 1-4). In addition, it is believed that GPCR-mediated generation of reactive oxygen species (ROS) mediate RTK transactivation by inactivating protein tyrosine phosphatases (PTPs) that act on RTKs, (Figure 1-4) (83).

In contrast to transactivation of RTKs, classical GPCR-mediated stimulation of the second messenger-dependent protein kinases (i.e. PKA and PKC) also accounts for several instances of ERK/MAPK activation (Figure 1-4). For example PKA is known to phosphorylate the Ras-family GTPase Rap-1, perhaps through Src family tyrosine kinases, thereby activating B-Raf and stimulating ERK (84-86). It is also possible that
binding of cAMP to the Rap-1 GEF, Epac, activates Rap-1 and leads to activation of B-Raf (87). Evidence also exists suggesting that PKC can activate ERK, apparently via direct phosphorylation of Raf-1 (88).

Figure 1-4. GPCRs activate the ERK/MAPK cascade through several mechanisms. GPCRs most commonly activate the ERK/MAPK cascade via receptor tyrosine kinase (RTK) transactivation. As diagrammed here, this has been shown to occur via activation of membrane metalloproteases of the ADAM family and subsequent autocrine/paracrine release of EGF receptor ligands (e.g. heparin-binding epidermal growth factor or HB-EGF) from the cell surface. This results in the recruitment and scaffolding of a Ras activation complex and classical activation of the ERK/MAPK cascade. In addition, GPCR-mediated generation of reactive oxygen species (ROS) contributes to RTK transactivation by inactivating protein...
tyrosine phosphatases (PTPs) that act on RTKs. Several mechanisms of GPCR-mediated ERK/MAPK activation that do not rely on RTK transactivation include the activation of Raf1 by the second messenger-dependent protein kinases PKA and PKC. These kinases have been shown to either directly (PKC) or indirectly (PKA) activate Raf1.

In addition to the pathways outlined in Figure 1-4, alternative mechanisms exist whereby GPCRs activate the ERK/MAPK signal transduction cascade. For example, integrin-based scaffolds at focal adhesions have been shown to link GPCRs with the ERK/MAPK pathway (82). Integrins, which exist as single transmembrane heterodimers, lack tyrosine kinase activity; therefore they act by scaffolding non-receptor tyrosine kinases such as the Ca\(^{2+}\) and cell adhesion-regulated focal adhesion kinase (FAK) family kinase PYK2 and the focal adhesion kinase (p125 FAK) which then activate ERK/MAPK similar to RTKs (Figure 1-5). Another mechanism is receptor endocytosis, which was shown to facilitate both RTK- and GPCR-mediated ERK/MAPK activation (89-91). Apparently a minority of GPCRs that did not internalize were also affected by endocytosis inhibitors (92), thus implying that a downstream component of the cascade must engage with the endocytic machinery (78). However, studies show that not all GPCRs are affected by endocytosis inhibitors and suggest the existence of additional pathways. For instance, the non-receptor tyrosine kinase c-Src is also implicated in ERK/MAPK activation by many GPCRs (77,93,94), many of which require the novel MAPK scaffold arrestin as discussed above Chapter 1.2.5 (Figure1-3) (52,95,96).
Figure 1-5. GPCRs activate the ERK/MAPK cascade via integrin-based scaffolds. GPCR-mediated PLC activation and subsequent release of Ca\(^{2+}\) from intracellular stores activates the calcium- and cell-adhesion regulated FAK family kinase, Pyk2, thereby promoting its association with focal adhesions. Once activated, numerous signaling proteins including Src, Csk, paxillin, and the Ras GEFs, Crk-C3G and Grb2-mSos, are recruited into the complex, promoting Ras activation and initiating the MAPK/ERK1/2 cascade. The focal adhesion kinase, p125FAK, is also recruited to focal adhesions, leading to tyrosine phosphorylation-dependent recruitment of Grb2-mSos and Ras activation. Adapted from (82).

At this point it is clear that GPCRs activate RTKs and the ERK/MAPK cascade through a variety of mechanisms. Surprisingly, recent studies have shown that RTKs
return the favor and use GPCR signaling molecules to transduce signals, and that RTK ligands themselves can transactivate GPCRs. In the case of RTKs utilizing GPCR signaling molecules, it has been shown that RTKs can directly engage heterotrimeric G proteins (i.e. G\textsubscript{ai}, G\textsubscript{as}, G\textsubscript{a13}, and G\textsubscript{b\gamma}) and arrestins to produce cellular responses such as mitogenesis, cell migration, anti-apoptosis, and clathrin-mediated endocytosis (79).

RTKs also signal through GPCR signaling pathways by transactivating GPCRs. Several reports have shown that this occurs through synthesis and secretion of GPCR ligands such as the CCR5 chemokine receptor ligand RANTES and the sphingosine 1 phosphate 1 (S\textsubscript{1P\textsubscript{1}}) receptor ligand S\textsubscript{1P} (97,98), as well as through a ligand-independent process involving formation of GPCR-RTK complexes and sometimes phosphorylation of the GPCR (79). For instance, studies using inverse agonists for S\textsubscript{1P\textsubscript{1}} and LPA\textsubscript{1} (LPA\textsubscript{1}) receptors have shown that PDGF and neurotrophin signaling requires G protein subunits provided by constitutively active S\textsubscript{1P\textsubscript{1}} and LPA\textsubscript{1} receptors, respectively (99-101). Moreover, RTK ligand-mediated signaling has been shown to depend on the phosphorylation of certain GPCRs including the PACAP type 1 (PAC1) receptor, the \textgreek{b}_2AR, and the S\textsubscript{1P\textsubscript{1}} receptor (79). Taken together, these results suggest that GPCR transactivation by RTK ligands is important for distinct aspects of RTK signaling, including those associated with malignant phenotypes such as proliferation, migration, and anti-apoptosis. It follows that novel therapeutic approaches which target RTK-GPCR crosstalk at the level of GPCR signaling (i.e. GPCR antagonists or inverse agonists) could be very effective and produce less pronounced side effects than traditional approaches which directly inhibit RTK signaling.
1.2.7 GPCR Regulation

1.2.7.1 Canonical GPCR Regulation

It is no surprise that GPCRs are closely regulated given the enormous diversity of GPCR ligands, receptor sequences, signal transduction pathways, and physiological responses. Moreover, attenuation of GPCR signaling following activation is necessary to maintain signaling homeostasis and circumvent receptor overstimulation. As introduced above, the intrinsic GTPase activity of the Gα subunit (i.e. $K_{cat} \sim 4$/min) inhibits further G protein-mediated signal transduction by favoring re-association of the GDP-bound Gα subunit with the Gβγ subunit. Importantly, GAPs (or RGS proteins) have been identified that function to increase the rate of GTP hydrolysis nearly 100-fold (i.e. $K_{cat} \sim 0.5$/sec at 4°C), thereby accelerating formation of the inactive G protein heterotrimer (102).

In addition to RGS proteins, GPCR activation is also attenuated via a canonical regulatory process involving attenuation of receptor responsiveness, known as receptor desensitization. According to the canonical model of GPCR desensitization, GPCR responsiveness is attenuated in a stepwise manner incorporating rapid receptor phosphorylation and arrestin recruitment, receptor endocytosis via clathrin-coated pits, and either recycling back to the plasma membrane for additional rounds of signaling (i.e. resensitization) or trafficking to lysosomes for degradation (i.e. down-regulation) (Figure 1-6). As well, these processes are kinetically distinguishable and occur within seconds (phosphorylation), minutes (internalization), or hours (down-regulation), the extent of which may vary according to receptor structure and cellular environment (103).

The rapid phosphorylation of GPCRs and subsequent recruitment of arrestins is responsible for the initial stage of receptor desensitization (Figure 1-6). This step, which
serves to initially uncouple GPCRs from heterotrimeric G proteins, is typically mediated via two classes of serine/threonine protein kinases which include 1) the second messenger-activated protein kinases PKA and PKC and 2) the GPCR kinases (GRKs 1-7). Importantly, initial uncoupling from G proteins due to phosphorylation is not necessarily dependent on arrestins since PKA and PKC are not known to recruit arrestin. Extensive studies have determined that GPCR desensitization can occur via agonist-dependent and -independent mechanisms. Agonist-dependent receptor desensitization (i.e. homologous desensitization) decreases a cell’s response to only that agonist to which the cell has been exposed and is, therefore, receptor-specific. In contrast, agonist-independent desensitization (i.e. heterologous desensitization) of a specific GPCR can occur in the absence of agonist activation, and results from activation of second messenger-dependent protein kinases (i.e. PKA and PKC) downstream of other receptors. Interestingly, both second messenger-dependent protein kinases and GRKs contribute to homologous GPCR desensitization and their relative contributions are dependent on agonist concentration, cell type, and the type of GPCR (103,104). For example, the second messenger dependent protein kinase PKA is responsible for desensitizing the β2AR at low agonist concentrations; whereas GRKs are responsible for β2AR desensitization at high agonist concentrations (105).

In addition to the prototypic βAR/adenylyl cyclase pathway, numerous studies have addressed the regulation of PLCβ-coupled GPCRs. It is clear that a large number of PLCβ-coupled receptors are potential substrates for PKC, as determined by phorbol ester activation of PKC (106). However, for most of these receptors the phosphorylation is not affected by PKC inhibitors. Thus it appears that for the majority of GPCRs, PKC was
either not involved or partly responsible for agonist-induced phosphorylation. Interestingly, a considerable number of PLCβ-coupled receptors are phosphorylated in response to agonist activation in a manner consistent with the action of a receptor-specific kinase (106). In these studies homologous receptor phosphorylation was rapid (10-300sec), typically required high agonist concentrations, and was distinct from second-messenger regulated kinases. Some of these kinases were identified and included the βAR kinase (GRK) and casein kinase 1-α. Significantly, a majority of these receptor-specific kinases have not been identified and the elucidation of these kinases remains an intriguing research question.

Once internalized, the fate of a GPCR is determined via its interaction with any number of proteins such as SNX-1, NSF-1, and GASP which function to actively sort GPCRs to lysosomes for proteolysis or to recycling endosomes for resensitization (107)(Figure 1-6). Moreover, these proteins and pathways are specific for certain GPCRs since swapping defined cytoplasmic regions of GPCRs is sufficient to completely alter their trafficking.
Figure 1-6. Canonical model of GPCR regulation. Modeled after extensive studies exploring the regulation of β-adrenergic receptors, attenuation of GPCR responsiveness (i.e. desensitization) is thought to occur via a process involving rapid receptor phosphorylation and arrestin recruitment, receptor endocytosis via clathrin-coated pits, and either recycling of receptors back to the plasma membrane for another round of signaling (i.e. resensitization) or trafficking to lysosomes for proteolysis (i.e. down-regulation). The initial stage of GPCR desensitization which is responsible for uncoupling the receptor from activated G proteins is mediated via 1) phosphorylation of the receptor by a GPCR kinase (GRK) followed by arrestin binding or 2) receptor phosphorylation by the second messenger-activated protein
kinases cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC). Moreover, receptor phosphorylation can be agonist-dependent (homologous, mediated via GRKs or PKA/PKC) or agonist–independent (heterologous, mediated via PKA/PKC). Once internalized, GPCRs interact with sorting proteins such as SNX-1, NSF-1, and GASP which function to traffic the receptors to lysosomes for proteolysis or to recycling endosomes for resensitization.

1.2.7.2 Emerging Evidence for Growth Factor Receptor-Mediated Regulation of GPCRs

As discussed above, the signaling repertoire of GPCRs includes the activation of the ERK/MAPK signaling cascade via numerous mechanisms, most commonly through transactivation of RTKs (82). Although it is known that RTKs ‘hijack’ GPCRs and signaling proteins such as heterotrimeric G proteins and arrestins to exert some of their affects (Chapter 1.2.6) (79), data from a small set of GPCRs supports a novel regulatory paradigm whereby RTK activation modulates GPCR signaling. Recently defined as ‘counter-regulation’, RTK activation has been shown to profoundly affect the signaling of several GPCRs including the β1-, β2-, and α1B-adrenergic receptors (108-112), as well as the 5-HT2C receptor (113). As shown in Figure 1-7 for the β2AR endogenously expressed in DDT1 MF-2 hamster smooth muscle cells, activation of the insulin receptor promotes phosphorylation of the C-terminal tail at several loci including three events directly mediated by the insulin receptor (Tyr350, Tyr354, and Tyr364) and two events mediated by Akt (Ser345 and Ser346). It has been determined that some of these phosphorylation events not only result in functional antagonism of cAMP accumulation
but facilitate receptor internalization. In addition, activation of the insulin-like growth factor-1 (IGF-1) receptor leads to decreased β2AR signaling, most likely due to phosphorylation within the i2 loop (111). Further studies in CHO cells transiently expressing the β1AR and cardiac myocytes endogenously expressing the β1AR show that IGF-1 treatment results in a rapid decrease in isoproterenol-induced cAMP, followed by internalization of the β1AR (108). Interestingly, growth-factor mediated regulation of βARs has profound physiological consequences and likely represents a novel regulatory mechanism that occurs between additional RTKs and GPCRs.

Figure 1-7. Receptor tyrosine kinases modulate GPCR signaling. Recent studies have uncovered a novel regulatory mechanism whereby growth factor-activated receptor tyrosine kinases (RTKs) regulate GPCR signaling. As shown for the β2AR, the activated insulin receptor directly phosphorylates Tyr350, Tyr354, and Tyr364
within the C-terminus of the receptor (110,114). The β2AR is also phosphorylated on Ser345 and Ser346 following insulin receptor-mediated activation of phosphatidylinositol 3-kinase (PI3K), 3-phosphoinositide-dependent protein kinase-1 (PDK1), and Akt (114). Some of these phosphorylation events have been shown not only to result in functional antagonism of cAMP accumulation but facilitate receptor internalization. A recent report also shows that activation of the insulin-like growth factor-1 (IGF-1) receptor abolishes β2AR-mediated cAMP accumulation, probably through phosphorylation of Tyr132 and Tyr 141 within the i2 loop. Likewise, additional studies show that β1AR, α1bAR, and 5-HT2C signaling events are modulated by activated RTKs.

1.3 5-Hydroxytryptamine Receptors

1.3.1 Introduction

The biogenic indoleamine neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) was first identified as a vasoconstrictor in defibrinated blood at the Cleveland Clinic Foundation in 1948 (115). Biosynthesis of 5-HT is known to occur through the hydroxylation of tryptophan by tryptophan hydroxylase to form 5-hydroxytryptophan (5-HTP), followed by the subsequent decarboxylation of 5-HTP by the aromatic L-amino acid decarboxylase (AADC). The majority of the body’s 5-HT (~90%) is found in the enterochromaffin cells of the gastrointestinal tract where it regulates gastrointestinal motility, with the remainder of the body’s 5-HT stored in platelet dense granules where its release promotes platelet aggregation. However, 5-HT cannot cross the blood-brain barrier, and must be synthesized by raphe neurons in the central nervous system (CNS).
5-HT is involved in numerous physiological and behavioral systems and, consequently, has been the focus of extensive research efforts. For instance, 5-HT influences cardiovascular regulation, respiration, thermoregulation, circadian rhythm entrainment, sleep-wake cycle, appetite, aggression, sexual behavior, sensorimotor reactivity, pain sensitivity, and learning. Pharmacological regulation of serotonin function has been found to influence a range of psychiatric disorders including depression, anxiety, schizophrenia and anorexia nervosa. In addition, a range of impulse-related disorders and personality features have been associated with alterations of serotonin function including aggression, substance abuse, gambling, obsessive control, and attention deficit disorder (reviewed in (116)).

In mammals, 5-HT mediates its actions through at least 15 receptor subtypes contained within seven major serotonin receptor families: 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆, and 5-HT₇. Except for the 5-HT₃ receptor, which is a ligand-gated ion channel, all other 5-HT receptors are members of the group A family of rhodopsin-like GPCRs and are very highly conserved (117,118). Interestingly, 5-HT receptors have also been identified in nematodes and other invertebrates with relatively simple nervous systems with most showing similarity to mammalian 5-HT₁ and 5-HT₇ receptors; whereas bacteria and single-celled eukaryotes such as yeast are devoid of 5-HT receptors. In fact, some invertebrates such as Caenorhabditis elegans have multiple receptor subtypes thus suggesting an early evolutionary push for diverse 5-HT receptor-mediated signaling (119).

The alignment of the primary sequence of vertebrate 5-HT receptors reveals several highly conserved residues which, when mutated, can have functional implications
(Figure 1-8). In TM1, Asn1.50 is conserved among 104 vertebrate 5-HT receptors, followed by Val1.53 which is conserved in every receptor except for 5-HT6 receptors which have a conservative substitution to Leu. In TM2, Asp2.50 is completely conserved and has been shown to have variable effects on ligand affinity and G protein coupling and mediate interactions with TM7 (120-122). Also in TM2, Ser2.45 and Val2.57 are completely conserved among 5-HT receptors. In TM3 the D-R-Y motif (Asp3.49, Arg3.50, Tyr3.51), which is present in over 70% of GPCRs and is thought to be crucial for maintaining GPCRs in an inactive state (i.e. the ‘ionic lock’) (27,35), is conserved in all but one 5-HT receptor (i.e. the pufferfish 5-HT7 receptor). In addition, Asp3.32 is conserved among 5-HT receptors and is thought to anchor the amine moiety of 5-HT and other ligands (123). Ser3.39, Ile3.40, and Ile3.46 are also completely conserved in TM3. In TM4, only Trp4.50 is conserved and has been shown to significantly affect 5-HT potency and efficacy (124). In TM5, Pro5.50 and Tyr5.58 are completely conserved in which Pro5.50 is thought to be related to the existence of an underwound π-helix. In addition, mutations at the conserved Ser5.43, Phe5.47, and flanking residue Phe5.48 selectively affect ligand affinity, while mutations at Phe5.47 and Phe5.48 reduce potency and efficacy of 5-HT-stimulated PIP2 hydrolysis (125). TM 6 and TM7 are two of the most conserved helices for 5-HT receptors and include several residues such as Trp6.48 and Phe6.52 whose aromatic side chains are important for ligand binding at the 5-HT2A receptor (124,126). Moreover, single and reciprocal mutagenesis studies at conserved Glu6.30 and Arg3.50 support the ‘ionic lock’ between TM6 and TM3 that might mediate receptor activation (35). In TM7, mutations at Trp7.40 and Tyr7.43 decrease agonist binding and activity (124). As shown for TM2, Asn7.49 is thought to interact with
Asp2.50 and affect receptor activation (122). Moreover, the completely conserved Tyr7.53 located within the N-P-X-X-Y motif is likely to be important for 5-HT receptor activation since mutation leads to a constitutively active 5-HT<sub>2C</sub> receptor (127). As expected, almost all of the highly conserved residues have large effects on agonist binding and receptor signaling.

1.3.2 5-Hydroxytryptamine Receptor Activation and Signal Transduction

As discussed above, numerous conserved residues are implicated in 5-HT receptor signaling. However, the mutation of several key residues has been shown to activate 5-HT receptors in the absence of agonist (Figure 1-8). For instance, mutating position 6.34 in the C-terminal region of the i3 loop of 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors produces constitutively active mutants (118,123). More recent studies have shown that disrupting the ‘ionic lock’ between the conserved D-R-Y motif in the cytoplasmic region of TM3 and a glutamate residue in the cytoplasmic region of TM6 promotes constitutive activity of 5-HT<sub>2A</sub> receptors (35). In the same study it was also apparent that minor changes in the side chain volumes of Cys6.34, Val6.36, and Val6.40 located at the interfaces between TMs 6 and 7 and between TMs 5 and 6 promote constitutive receptor activation. As mentioned above, the completely conserved Tyr7.53 located within the N-P-X-X-Y motif is also likely to be important for 5-HT receptor activation since its mutation causes constitutive activity in 5-HT<sub>2C</sub> receptors (127). These studies imply that the inactive state of 5-HT receptors, with an emphasis on 5-HT<sub>2A</sub> receptors, is stabilized by ionic and hydrophobic interactions mainly involving residues located within the intracellular half of TM6.
Once activated, 5-HT receptors primarily couple to G\textsubscript{ai/o} (5-HT\textsubscript{1}) and G\textsubscript{as} (5-HT\textsubscript{4,6,7}) G proteins to modulate adenylate cyclase activity, as well as the Gaq (5-HT\textsubscript{2}) G protein to modulate PLC\textbeta\textsubscript{1} activity (reviewed in(117)). It is now realized that each 5-HT receptor couples to a wide variety of secondary signal transduction pathways, some of which are shared by several subtypes, and others that are unique (119). The primary and secondary signal transduction pathways for the 5-HT\textsubscript{2A} receptors will be discussed in detail below.

Figure 1-8. Many of the conserved residues in 5-HT receptors have roles in receptor signaling and activation. This snake diagram of the rat 5-HT\textsubscript{2A} receptor identifies residues that are conserved across all vertebrate 5-HT receptors (red filled circles). Mutagenesis studies have showed that many of these conserved residues are
important for 5-HT receptor signaling (yellow filled circles) and activation (green filled circles). Highlighted is the ‘ionic lock’ (large dashed line) that is implicated in maintaining the receptor in an inactive state.

1.3.3 5-Hydroxytryptamine 2A (5-HT$_{2A}$) Receptors: Identification, Localization, and Relevance for Study

The existence of multiple 5-HT receptors, originally described as M (morphine) and D (dibenzylamine) tryptamine sites, were first identified from organ bath studies over 50 years ago (128-130). Subsequent pioneering radioligand studies identified multiple 5-HT receptor classes (131) one of which was labeled with the neuroleptic $[^3]$H]spiperone (132). Shortly thereafter, Peroutka and Snyder (133) proposed the existence of two 5-HT sites, 5-HT$_1$ and 5-HT$_2$, one of which (i.e. the 5-HT$_2$ site) corresponded pharmacologically to Gaddum’s D tryptamine site and was labeled with $[^3]$H]spiperone. The 5-HT$_2$ site became known as the 5-HT$_{2A}$ receptor and was later cloned by Pritchett et al. (134). In the years following, the widespread use of radioligands, functional readouts, and molecular biology saw the rapid identification of additional 5-HT receptors including the 5-HT$_{2C}$ (originally named 5-HT$_{1C}$) and 5-HT$_{2B}$ receptors (123), among others (e.g. 5-HT$_{1A}$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$, and 5-HT$_7$) (117).

Of all the 5-HT receptors, the G$\alpha$q-coupled 5-HT$_2$ receptor class (5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$) is of particular interest. These receptors mediate many physiological functions of 5-HT in the periphery and central nervous system (CNS) including smooth muscle contraction, platelet aggregation, control of endocrine secretion, feeding behavior, mood, pain, sleep, thermoregulation, learning, and memory. Additionally, these receptors
have been implicated in several CNS disorders such as schizophrenia, depression, mania, and anxiety. Importantly, 5-HT$_2$ receptors are targets for many drugs including, but not limited to, atypical antipsychotics, anxiolytics, antidepressants, and most hallucinogens (123,135).

5-HT$_{2A}$ receptors, in particular, are localized to somatodendritic and axonal compartments within many CNS brain regions including the cerebral cortex, hippocampus, basal ganglia, olfactory tubercle, amygdala, diencephalon, cerebellum, brainstem, and spinal cord (136,137). 5-HT$_{2A}$ receptors are the predominant 5-HT receptors expressed in the cerebral cortex and are enriched in apical dendrites, and to a lesser extent in asymmetric synapses and dendritic spines of pyramidal neurons (136,138-140). Consequently, the 5-HT$_{2A}$ receptor is an especially interesting target for CNS disorder therapies since localization to proximal apical dendrites highlights its importance in controlling dendritic current propagation to the pyramidal cell body (141,142).

Specifically, 5-HT$_{2A}$ receptors are thought to modulate information flow along pyramidal cells by enhancing spontaneous excitatory postsynaptic potentials and currents (143). It has been proposed by at least one group that the inability of pyramidal neurons to attenuate firing in the absence of stimuli, either due to hyperactive 5-HT$_{2A}$ receptors or increased 5-HT$_{2A}$ receptor expression in apical dendrites, is responsible for psychotic behavioral states occurring in the positive acute phase of schizophrenia (139). It follows that atypical antipsychotics (e.g. clozapine) are thought to mediate their therapeutic actions, at least in part, by antagonizing 5-HT$_{2A}$ receptors and decreasing dendritic targeting of 5-HT$_{2A}$ receptors in cortical pyramidal neurons (144,145).
Early pharmacological evidence, in addition to the neuroanatomical and electrophysiological evidence, suggested that 5-HT receptors, and 5-HT\textsubscript{2A} receptors in particular, are important therapeutic targets for treating psychotic illness such as schizophrenia. As early as 1954, it was proposed that 5-HT was involved in the pathogenesis of schizophrenia due to its structural similarity with the prototypical hallucinogen lysergic acid diethylamide (LSD) (146). In fact, it is now realized that 5-HT receptors (specifically 5-HT\textsubscript{2A}) are the site of action of most (147), but not all (148) hallucinogens which function as 5-HT\textsubscript{2A} receptor agonists. Subsequent studies showing that the neuroleptic spiperone bound a serotonin receptor (later defined as the 5-HT\textsubscript{2A} receptor (123,132)), and that the atypical antipsychotic clozapine was a high affinity antagonist of 5-HT\textsubscript{2A} receptors (149), highlighted 5-HT\textsubscript{2A} receptors as an important target for combating psychotic illness. In fact, drug discovery efforts based on a clozapine-like pharmacology (i.e. drugs with higher affinity for 5-HT\textsubscript{2A} receptors than dopamine D\textsubscript{2} receptors) have yielded novel atypical antipsychotics including olanzapine, ziprasidone, zotepine, and quetiapine. These new compounds, devoid of the side effect profile of clozapine, represent advancements in the treatment of psychotic illness; however, clozapine still remains the most effective treatment for schizophrenia (141). Additionally, basic research findings support the relevance of 5-HT\textsubscript{2A} receptors in antipsychotic drug action. Specifically, 5-HT\textsubscript{2A} selective antagonists such as MDL100907 are able to block amphetamine- and PCP-induced hyperlocomotion, as well as block MK801-induced disruptions in pre-pulse inhibition (150).

5-HT\textsubscript{2A} receptors are also expressed in peripheral tissues including platelets where they facilitate platelet aggregation (151) and in many vascular (i.e. rat aorta and jugular
vein (152)) and non-vascular (i.e. rat uterus and guinea pig trachea (153)) smooth muscle preparations where they mediate contraction (reviewed in (154)). 5-HT_{2A} receptors are also shown to contribute to part of the contractile effect of 5-HT in guinea pig ileum as well as increased capillary permeability (154,155).

In summary, 5-HT_{2A} receptors have been shown to be essential for mediating a large number of physiologic processes in peripheral and central tissues including platelet aggregation, smooth muscle contraction, and the modulation of mood and perception. Furthermore, their dysregulation has been associated with a large number of diseases including schizophrenia, which is supported by studies showing that 5-HT_{2A} receptors are the site of action of most, but not all hallucinogens which function as 5-HT_{2A} receptor agonists. Accordingly, many drugs such as the atypical antipsychotics mediate their actions, at least in part, by antagonizing 5-HT_{2A} receptors. Thus, activation of 5-HT_{2A} receptors produces hallucination, whereas blockade of 5-HT_{2A} receptors is therapeutic. From this perspective it is imperative that researchers further probe 5-HT_{2A} receptor function in order to gain an increased understanding of the molecular and cellular mechanisms governing 5-HT_{2A} function, thereby facilitating a more efficient and focused approach toward treating 5-HT_{2A} receptor dysfunction. Since the cloning of the 5-HT_{2A} receptor, steady progress has been made in understanding receptor signaling and regulation and will be detailed below.

1.3.4 5-HT_{2A} Receptor-Mediated Signal Transduction

5-HT_{2A} receptors transduce extracellular signals via primary and secondary signal transduction cascades which incorporate a large variety of G proteins and effectors
5-HT$_{2A}$ receptors primarily signal through Gaq/11 proteins to PLCβ which hydrolyzes the membrane phospholipid PIP$_2$ into the second messengers IP$_3$ and DAG (156-159). Increases in DAG are known to stimulate PKC which results in the phosphorylation of numerous substrates on Ser or Thr residues thus altering their function (160), while rapid increases in the labile second messenger IP$_3$ promotes Ca$^{2+}$ release from intracellular stores via stimulation of IP$_3$-gated Ca$^{2+}$ channels on the endoplasmic reticulum (Figure 1-5). These increases in intracellular Ca$^{2+}$ have been associated with numerous functions including activation of PKC and a variety of ion channels (e.g. Ca$^{2+}$ and K$^+$ channels) (119). Importantly, 5-HT$_{2A}$ receptor-mediated increases in intracellular Ca$^{2+}$ are also known to activate CaM, which is considered to be the major or primary signaling target of Ca$^{2+}$ signaling in cells. This is supported by 1) the presence of two CaM-binding sites in the i2 loop and C-terminus which can abrogate G protein coupling (161), 2) the dependence of agonist-induced upregulation of 5-HT$_{2A}$ receptors on CaM and Ca$^{2+}$ CaM-dependent kinase 2 (162), 3) the 5-HT$_{2A}$ receptor induces increases in cAMP via CaM in A1A1 cells (163), and 4) the 5-HT$_{2A}$ receptor induces cyclooxygenase 2 mRNA expression via a CaM-dependent pathway in renal mesangial cells (164,165).

5-HT$_{2A}$ receptors also initiate several secondary signal transduction cascades, as shown in Figure 1-9. For example, 5-HT$_{2A}$ receptors 1) activate phospholipases such as PLD and PLA$_2$ (166,167), 2) inhibit and stimulate cAMP formation (163,168), 3) induce tyrosine phosphorylation of Janus kinase 2 (Jak2) and STAT3 thereby leading to STAT3 nuclear translocation (169), 4) regulate dynamics of the actin cytoskeleton through Rho (170), 5) stimulate and inhibit nitric oxide (NO) synthesis, and 6) regulate a variety of
transport processes including activation of the Na\(^+\)/H\(^+\) exchanger 1 (NHE1) (119). Similar to other GPCRs, 5-HT\(_{2A}\) receptors are also known to activate the ERK/MAPK pathway (171,172), which explains data showing that 5-HT\(_{2A}\) activation mediates vascular smooth muscle contraction (173), cellular proliferation (174) and results in the induction of the early response genes cyclo-oxygenase-2 and Egr-1 (165). Recent studies have examined this in great detail and have identified several signaling intermediates linking the 5-HT\(_{2A}\) receptor to ERK/MAPK activation (Figure 1-9). In vascular smooth muscle cells, 5-HT\(_{2A}\) activates ERK via transactivation of receptor tyrosine kinases (173) and activation of MEK1 (175), and NHE1 (176). Moreover, Greene et al. (177) have reported in rat renal mesangial cells that 5-HT\(_{2A}\) receptors activate ERK via generation of ROS (i.e. H\(_2\)O\(_2\)). Specifically, 5-HT\(_{2A}\) activation initiated a signaling cascade progressing from PLC\(\beta\) activation and PKC activation to the generation of ROS through a NAD(P)H oxidase enzyme (Figure 1-9). As mention previously, generation of ROS species inactivates PTPs, thereby promoting RTK signaling. A later study in these same cells showed that 5-HT\(_{2A}\)-mediated proliferation occurred via transactivation of the EGF receptor which occurred through activation of tumor necrosis factor \(\alpha\)-converting enzyme (TACE, ADAM17) and subsequent ectodomain shedding of the EGF receptor ligand HB-EGF (178). These studies clearly show that 5-HT\(_{2A}\) receptors can produce a variety of signals through both primary and secondary effector pathways.
Figure 1-9. **Primary and secondary 5-HT$_{2A}$-mediated signal transduction pathways.**

In this diagram, activated 5-HT$_{2A}$ receptors signal primarily through phospholipase C$\beta$ (PLC$\beta$) which hydrolyzes the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP$_2$) into the second messengers inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). The labile second messenger IP$_3$ then promotes Ca$^{2+}$ release from intracellular stores via stimulation of IP$_3$-gated Ca$^{2+}$ channels on the endoplasmic reticulum. Increases in DAG, along with intracellular Ca$^{2+}$, have been shown to activate protein kinase C (PKC). In addition, Ca$^{2+}$ goes on to activate calmodulin and various ion channels. As shown in the extreme right and left portions of the figure, 5-HT$_{2A}$ receptors also mediate their actions by activating a variety of secondary pathways. One such pathway is the activation of the ERK/MAPK cascade, which explains many effects of 5-HT including mitogenesis and early response gene expression. This is thought to occur through a variety of pathways and intermediates such as membrane metalloprotease-mediated
transactivation of the epidermal growth factor (EGF) receptor, generation of reactive oxygen species (ROS), and activation of NHE1 and MEK1.

1.3.5 5-HT₂A Receptor Regulation: Rapid Desensitization and Internalization

Studies over the past two decades have provided some insight into the regulation of 5-HT₂A receptors (Figure 1-10). Interestingly, a consensus has emerged suggesting that 5-HT₂A receptors are not regulated in the classical way depicted in Chapter 1.2.7 (Figure 1-6). Instead, 5-HT₂A receptors are unique in that they 1) are not detectably phosphorylated by GRKs or PKC following agonist activation, despite undergoing desensitization; 2) are arrestin-insensitive; and 3) are internalized and down-regulated following antagonist treatment (i.e. paradoxical regulation). As discussed below it is clear that much more work is needed in order to adequately explain 5-HT₂A receptor regulation.

Extensive studies focusing on the (GRK)-arrestin pathway and the second messenger-dependent protein kinase PKA and PKC pathways suggest that direct GPCR phosphorylation remains the predominant mechanism for rapidly attenuating the signaling of many GPCRs (179,180). Additional kinases have also been shown to phosphorylate GPCRs and it is likely that many yet-to-be discovered kinases regulate GPCR signaling (181). Studies addressing rapid 5-HT₂A receptor regulation by kinases are ambiguous and lack direct evidence of receptor phosphorylation. Specifically, several studies demonstrate that PKC modulates 5-HT₂A receptor signaling in vivo and in vitro. Early studies (182) showed that activation of PKC by phorbol dibutyrate inhibited 5-HT₂A-mediated signaling. Many subsequent studies in a variety of cellular contexts have
replicated these observations (183-188). Moreover, recent work with the PKCγ knock-out mouse showed increased DOI-induced head-twitch (189). However, inhibition of PKC failed to alter 5-HT2A desensitization in PS200 cells (190), as did mutation of all intracellular PKC consensus phosphorylation sites of 5-HT2A receptors expressed in HEK293 cells (191). In addition to PKC, recent reports suggest that calmodulin-dependent protein kinase II and GRK2 and GRK3 regulate 5-HT2A signaling (188,192), although the role of GRKs seems to be cell type-specific (193). From these studies it is clear that selected kinases modulate 5-HT2A receptor function, although the site(s) of action and their mechanisms remain unknown (Figure 10).

Receptor phosphorylation, in addition to rapidly attenuating agonist activation, is thought to recruit arrestins which function to further uncouple receptors from G proteins and tether receptors to the internalization machinery via interactions with both clathrin and the adaptor protein AP2 (194,195). Although it is known that 5-HT2A receptor internalization and trafficking to early endosomes occurs via clathrin-coated pits and requires dynamin (193,196-198), the role of arrestins is unknown. For instance, purified arrestin2 and arrestin3 are known to interact with the i3 loop of the 5-HT2A receptor in vitro and 5-HT2A receptors colocalize with arrestin2 and arrestin3 in some, but not all, cortical neurons (199). However, studies in HEK293 cells suggest that arrestin is not required for 5-HT2A receptor desensitization, internalization, and resensitization (193,198). Notably, this ‘arrestin insensitivity’ was not found in all cell types (i.e. C6 glioma) and could be rescued with the constitutively active arrestin mutant Arr2-R169E (200). Thus, the role of arrestins in 5-HT2A internalization and desensitization remains unclear and is cell-type specific. However, it is likely that the lack of agonist-induced
phosphorylation (172,190), which is supported by GRK insensitivity and the ability for a constitutively active arrestin to decrease signaling and promote internalization, accounts for the inability of arrestins to regulate 5-HT$_{2A}$ receptors.

1.3.6 5-HT$_{2A}$ Receptor Regulation: Down-Regulation and Resensitization

Although proteins responsible for trafficking of GPCRs to either lysosomes for degradation (i.e. down-regulation) or recycling endosomes for trafficking back to the membrane (i.e. resensitization) have been identified for other GPCRs, the machinery governing post-endocytic sorting of 5-HT$_{2A}$ receptors is unknown (Figure 1-10) (107). However, like most GPCRs, 5-HT$_{2A}$ receptors are down-regulated following prolonged agonist exposure in vivo and in vitro, albeit by an unknown mechanism (reviewed in (201)). Surprisingly, in stark contrast to the classical regulatory paradigm, 5-HT$_{2A}$ receptors are paradoxically down-regulated by chronic antagonist treatment. This phenomenon was initially identified in vivo following chronic treatment with antidepressants (202,203), and has since been observed for several classes of antidepressants and antipsychotics (extensively reviewed in (201)). Several mechanisms such as transcriptional regulation have been proposed to explain such behavior, although the majority of the evidence in vivo supports receptor degradation (204). Significantly, since 5-HT$_{2A}$ receptor antagonists have been shown to induce receptor internalization (145,196,197), it is possible that antagonist-induced internalization underlies the paradoxical regulation of 5-HT$_{2A}$ receptors (205).

Similar to circumstances surrounding 5-HT$_{2A}$ receptor down-regulation, the 5-HT$_{2A}$ receptor is known to undergo resensitization but the mechanism(s) remains
unknown. Clear cell-type specific mechanisms have been reported, as evidenced by the function of receptor internalization in the resensitization of 5-HT$_{2A}$ receptors in C6 glioma cells, but not in HEK293 cells (193). Interestingly, blocking internalization with a dominant negative mutant of dynamin, which attenuated resensitization of β$_2$ARs, actually potentiated 5-HT$_{2A}$ resensitization in HEK 293 cells suggesting the existence of a novel cell-surface mechanism. This finding is not entirely surprising given recent evidence of cell-surface regulation of β$_2$ARs (206). Taken together, clear evidence for a unifying regulatory mechanism of 5-HT$_{2A}$ receptor regulation is lacking. Therefore it seems likely that 5-HT$_{2A}$ receptors are regulated by other means including via receptor-interacting proteins.
Figure 1-10. The canonical GPCR regulatory model as applied to our current understanding of 5-HT_{2A} receptor regulation. Although 5-HT_{2A} receptors share some regulatory behaviors consistent with the canonical model of GPCR regulation (e.g. agonist-mediated desensitization and internalization), the precise mechanisms whereby 5-HT_{2A} receptors are regulated remain largely unknown. In addition, 5-HT_{2A} receptors exhibit some regulatory behaviors that are inconsistent with prototypical GPCRs (e.g. paradoxical regulation and arrestin independence). In this diagram the exact role of kinases is unknown (represented by dashed line) since numerous studies in a variety of cellular contexts have identified several kinases as being important for receptor desensitization including PKC, GRK2 and GRK3, calmodulin-dependent kinase II α (CamKIIα), and p90 ribosomal S6 kinase 2 (RSK2). However, the literature implicating PKC and GRKs is conflicted and has not provided evidence of direct receptor phosphorylation. Likewise, the data supporting the role of arrestins in 5-HT_{2A} receptor desensitization and internalization are inconsistent (represented by dashed line), despite the ability of a constitutively active arrestin mutant (CA-arrestin R169E) to promote receptor internalization and desensitization. This ‘arrestin insensitivity’ of 5-HT_{2A} receptors is most likely explained by the lack of receptor phosphorylation. Although proteins responsible for trafficking of GPCRs to either a lysosomes for degradation or recycling endosomes for resensitization have been identified for other GPCRs, the machinery governing post-endocytic sorting of 5-HT_{2A} receptors remains unknown.
1.3.7 5-HT$_2A$ Receptor Interacting Proteins

1.3.7.1 Overview

GPCRs not only interact with heterotrimeric G proteins, but also with accessory proteins called GPCR interacting proteins. These proteins, which include other transmembrane proteins (e.g. GPCRs and receptor activity modifying proteins or RAMPs) and soluble proteins (e.g. glutamate receptor interacting protein or GRIP), have been shown to localize GPCRs to distinct cellular compartments, mediate GPCR trafficking to and from the plasma membrane, scaffold functional complexes, and fine-tune GPCR signaling (51).

5-HT$_2A$ receptors are similar with respect to other GPCRs in that they interact with numerous proteins including 1) microtubule-associated protein 1A (MAP1A) (207), 2) human polyomavirus JCV (208), 3) arrestins (199,200), 4) calmodulin (161), 5) PDZ-domain containing proteins including post-synaptic density protein-95 (PSD-95) (209), 6) caveolin-1 (171), 7) RSK2 (172), and 7) ADP-ribosylation factor 1 (ARF1) (210). Many additional interacting proteins have been identified (172,211,212)and those with profound effects on 5-HT$_2A$ receptor signaling and/or trafficking will be described in detail below (Figure 1-11).
Figure 1-11. 5-HT$_{2A}$ receptor interacting proteins and their sites of interaction. This diagram shows that 5-HT$_{2A}$ receptors interact with several proteins, wherein some interactions are localized to discrete sites on the receptor (boxed) and shown to profoundly modulate 5-HT$_{2A}$ receptor function (these are highlighted in the text). Abbreviations used are: ADP-ribosylation factor 1 (ARF1), post-synaptic density protein-95 (PSD-95), activin receptor-interacting protein 1 (ARIP1), synapse-associated protein 97 (SAP97), MAGUK p55 subfamily member 3 (MPP-3), vertebrate homolog of LIN7 (Veli-3), channel-interacting PDZ domain protein (CIPP), antioxidant protein 2 (AOP-2), multiple PDZ protein 1 (MUPP1), microtubule-associated protein 1A (MAP1A), p90 kDa ribosomal S6 kinase 2 (RSK2), amyloid-beta precursor protein intracellular domain associated protein-1a (AIDA-1A), eukaryotic translation initiation factor 3, subunit 5 epsilon (eIF3S5), neurotrophic tyrosine kinase receptor type 3 isoform c precursor (NTRK3),
melanoma-associated antigen (MAAT1), paraoxonase 2 (PON2), nucleoside-diphosphate kinase 3 (NME3), NADH dehydrogenase (ubiquinone) 1 beta subcomplex (NDUFB10), protein phosphatase 5, catalytic subunit (PPP5C), and glutamine synthetase (GLUL).

1.3.7.2 PDZ domain-containing proteins including PSD-95

The extreme C-terminus of the 5-HT$_{2A}$ receptor contains a type I Post-synaptic density/Zonula occludens/Discs large (PDZ) motif which mediates interactions between the 5-HT$_{2A}$ receptor and a variety of PDZ domain-containing proteins with unknown functional consequences including activin receptor-interacting protein 1 (ARIP1), synapse-associated protein 97 (SAP97), MAGUK p55 subfamily member 3 (MPP-3), vertebrate homolog of LIN7 (Veli-3), channel-interacting PDZ domain protein (CIPP), antioxidant protein 2 (AOP-2), and multiple PDZ protein 1 (MUPP1) (211,212). Significantly, the 5-HT$_{2A}$ receptor C-terminus interacts with the post-synaptic density protein 95 (PSD-95) (209,212), and was shown to modulate both 5-HT$_{2A}$ signaling and trafficking in vitro and in vivo (209,213-215). Specifically, PSD-95 tethered 5-HT$_{2A}$ receptors to the plasma membrane thereby increasing signaling (209). In PSD-95 knockout mice, however, DOI-induced head twitch was significantly reduced compared to wild-type mice, perhaps as a result of increased receptor turnover (i.e. decreased receptor expression) in the cortex (214). Nonetheless, key experiments from both studies unequivocally showed that PSD-95 regulates 5-HT$_{2A}$ signaling in vitro and in vivo, probably due to scaffolding at the plasma membrane.
1.3.7.3 Caveolin-1

In addition to PSD-95, 5-HT\textsubscript{2A} receptors are known to interact with caveolin-1 (171), a multifunctional scaffold protein that is essential for caveolae formation and protein recruitment to these membrane invaginations rich in cholesterol and glycosphingolipids (216). Functionally, caveolins are known to control vesicular transport and cholesterol homeostasis, as well as modulate signal transduction by recruiting heterotrimeric G proteins, H-Ras, Src-family tyrosine kinases, and PLC\textbeta. Moreover, the existence of several phenotypes in caveolin knock-out mice highlights their importance (reviewed in (216)). The effect of caveolin-1 on 5-HT\textsubscript{2A} signaling was determined via siRNA-mediated knock-down of caveolin-1. In these studies Bhatnagar et al. (171) showed that inhibiting caveolin-1 expression abolished the 5-HT\textsubscript{2A}-mediated Ca\textsuperscript{2+} signaling; whereas over-expression of caveolin-1 increased its interaction with Gaq. Considering evidence that caveolin interacts with Gaq (217), and that 5-HT\textsubscript{2A} receptors and PLC\textbeta are enriched in lipid raft/caveolae membranes (171,218), it is likely that caveolin-1 scaffolds the receptor with the appropriate signal transduction components such as Gaq and PLC\textbeta in lipid rafts or caveolae thereby facilitating efficient signaling (215).

1.3.7.4 5-HT\textsubscript{2A} Receptor Interacting Proteins: p90 Ribosomal S6 Kinase2 (RSK2)

While direct evidence for 5-HT\textsubscript{2A} receptor phosphorylation remains elusive, recent work in this laboratory indicates that 5-HT\textsubscript{2A} receptor signaling is regulated by a novel pathway involving the p90 kDa ribosomal S6 family of serine/threonine kinases (p90RSK or RSK) which are downstream effectors of the ERK/MAPK cascade (172).
This finding was essential to the development of this thesis and will be described extensively below.

1.3.7.4.1 Identification and Activation of p90 Ribosomal S6 kinases (RSKs)

RSK2 was first identified as the major protein (MW=90,000) isolated from a crude extract from *Xenopus laevis* oocytes that phosphorylated the 40S ribosomal subunit protein S6 (219,220). Further purification of crude *Xenopus* egg extracts using DEAE-Sephascel chromatography by Erikson and colleagues (221) revealed two peaks of S6 kinase activity, S6KI (MW=90,000) and S6KII MW=92,000), the latter of which (S6KII) was extensively characterized since its activity toward S6 in vitro was similar to the maximal phosphorylation of S6 in vivo (219,221). Molecular clones were subsequently isolated from *Xenopus* cDNA libraries using oligonucleotide probes derived from the S6KII sequence and predicted two highly related proteins (~91%) of MW 83,000 with the unusual feature of two catalytic domains (222). Specifically, the N-terminal half of the molecule was determined to be related to the catalytic subunit of cAMP-dependent protein kinase (PKA), whereas the C-terminal half was found to be similar to the catalytic domain of phosphorylase b kinase. Homologs of S6KI and S6KII (renamed p90rsk or RSK1 and RSK2, respectively) were then cloned from mouse and chicken (223), rat, *Drosophila melanogaster* (224), and *Caenorhabditis elegans* (Nematode Sequencing Project, Wellcome Trust Sanger Institute, Hinxton, Cambridge). To date, 4 isoforms of RSK have been identified in humans: RSK1 (225), RSK2 (225), RSK3 (226), and RSK4 (227). RSKs are thought to be functionally redundant since RSK1-3 mRNA is expressed in every human tissue and brain region tested (228), although tissue-specific levels
suggest that RSKs may have specific roles. Specifically, RSK2 is most highly expressed in the cerebellum, frontal lobe, and occipital pole, with additional expression the cerebral cortex, medulla, spinal cord, temporal lobe, and putamen. RSK4 expression, however, is much lower than the other RSKs and is even undetectable in certain tissues (227).

As originally suggested by Jones et al. (222), RSKs are complex kinases composed of two functionally distinct kinase domains connected by a linker region and are flanked by N- and C-terminal tails containing distinct regulatory components (Figure 1-12). The N-terminal kinase domain (NTD), which is responsible for substrate phosphorylation, shows homology with the AGC family of kinases (e.g. PKA and PKC); whereas the C-terminal kinase domain (CTD), which autophosphorylates RSK during its activation, is homologous to the Ca\(^{2+}\)/calmodulin-dependent protein kinases (CamK) (229,230). RSK2 is maximally activated via a complex, sequential set of phosphorylation events that is initiated when the inactive ERK1/2 bound to the last C-terminal 15 amino acids of RSK2 (i.e. **Leu-Ala-Gln-Arg-Arg-Val-Arg-Lys-Leu-Pro-Ser-Thr-Thr-Leu**, residues conserved between RSK1 and 2 in bold) (231) is activated and phosphorylates Thr577 (human RSK2 numbering) within the CTD activation loop (Figure 1-12) (229,232). ERK also phosphorylates Ser365 and Ser369 within the linker region, wherein Ser369 phosphorylation is reported to increase subsequent N-terminal activation several fold. Next, the activated CTD autophosphorylates Ser386 within an AGC kinase hydrophobic motif (233), thereby recruiting PDK1 and increasing its catalytic activity several fold (234). After binding, PDK1 phosphorylates Ser227 within the NTD activation loop— a step that has been shown to be essential for RSK2 activation and is associated with human disease (see below) (235,236). Moreover, the NTD
autophosphorylates Ser737 to prevent RSK reactivation following dephosphorylation of the activating sites (231). Additional factors have been shown to affect RSK activation including p38 MAP kinase (i.e. through MAPK-activated kinase 2/3) (237), ERK5 MAP kinase (238), and the novel RSK kinases fibroblast growth factor receptor 3 (FGFR3) and Src tyrosine kinase family members Src and Fyn which phosphorylate Tyr529 to facilitate inactive ERK binding (239,240). Just recently, it was determined that FGFR3 binds Trp332 and phosphorylates RSK2 at Tyr707 within the autoinhibitory alphaL-helix thereby activating RSK2 (241).

![Figure 1-12. RSK structure and mechanism of activation. As detailed in this diagram, RSKs are complex kinases composed of two functionally distinct kinase domains connected by a linker region and are flanked by N- and C-terminal tails](image-url)
containing distinct regulatory components. The N-terminal kinase domain (NTD), which is responsible for substrate phosphorylation, belongs to the AGC family of kinases (e.g. PKA and PKC). The C-terminal kinase domain (CTD), which autophosphorylates RSK during its activation cycle, is homologous to the Ca$^{2+}$/calmodulin-dependent protein kinases (CamK). RSK is maximally activated via a complex, sequential set of phosphorylation events starting with binding and activation of ERK1/2. Once activated, ERK phosphorylates Thr577 (human RSK2 numbering) within the CTD activation loop and phosphorylates Ser365 and Ser369 within the linker region. Next, the activated CTD autophosphorylates Ser386 within an AGC kinase ‘hydrophobic motif’, thereby recruiting PDK1 and increasing its catalytic activity several fold. Activated PDK1 then phosphorylates Ser227 within the NTD activation loop-a phosphorylation event that is required for RSK substrate phosphorylation. In addition to substrate targets, the activated NTD autophosphorylates Ser737 within the ERK binding site to prevent RSK reactivation following dephosphorylation of the activating sites. Recent reports show that additional kinases including the fibroblast growth factor receptor 3 (FGFR3) and Src tyrosine kinase family members Src and Fyn promote RSK activation (239,240).

1.3.7.4.2 The Multiple Functions of RSK

RSK resides in many cellular compartments including the plasma membrane, cytoplasm, and nucleus (242) and therefore has numerous functions. In order to determine its biological significance, a massive effort has been undertaken within the last two decades to identify RSK substrates, which typically contain the basophilic minimum
consensus phosphorylation motif Arg-X-Arg-X-X-Ser/Thr (‘X’ denotes any amino acid) (243). An extensive literature reveals that RSKs are generally thought to regulate transcription and translation, the cell-cycle, and cell survival. Specifically, RSKs have been shown to regulate transcription via phosphorylation of transcription factors including cAMP-responsive-element binding protein (CREB) (244), serum response factor (SRF) (245), ATF4 which was shown to be required for proper osteoblast differentiation and function (246), ER81 which is strongly activated by MAPKs (247), estrogen receptor alpha (ERα) (248), NFATc4 (249), and NFAT3 (250). Notably, the involvement of RSK2 in CREB phosphorylation is uncertain since cells lacking functional RSK2 exhibit clear deficiencies in CREB phosphorylation and Fos induction (251); whereas some studies support a role for MSK1 in CREB regulation (252,253). In addition, RSKs modulate transcription via phosphorylating transcriptional co-activators (e.g. CREB binding protein (CBP) and P300 (254)) and transcription factor inhibitors (e.g. IκBα (255) and Mad1 (256)). In some cases RSK phosphorylates several immediate early gene products including c-Fos, JUN, and NUR77 to promote their stabilization, thereby ‘sensing’ RSK nuclear localization, signal duration, and signal strength (257).

RSK likely affects translation since upon activation it translocates to polyribosomes and phosphorylates ribosomal proteins (258). Indeed, recent work has established several mechanisms whereby RSK regulates translation including promoting mTOR signaling by phosphorylating and inhibiting the tuberin/hamartin complex (259), phosphorylating eukaryotic translation initiation factor 4B (eIF4B) thus increasing its association with eIF3 (260), phosphorylating ribosomal protein S6 at Ser235/236 thus stimulating cap-dependent translation (261), phosphorylating and inhibiting glycogen
synthase kinase 3 β (GSK3β) (262), and phosphorylating and inhibiting elongation factor 2 (EF2) kinase (263).

RSK is demonstrated to control cell cycle progression through several mechanisms involving different stages of the cell cycle. Specifically, RSK1 and RSK2 phosphorylate the cyclin-dependent kinase inhibitor p27KIP1 to promote G1 progression (264), RSK2 phosphorylates the inhibitory kinase Myt1 to promote G2-M progression via Cdc2 (265), and RSK1 and 2 are important for cytostatic factor-mediated metaphase arrest (242).

RSK-mediated cell-survival signaling has been shown to occur mainly through either inactivation of pro-apoptotic proteins or synthesis of anti-apoptotic proteins. For instance, RSK has been shown to phosphorylate, inactivate, and sequester within the cytoplasm the mitochondrial pro-apoptotic protein BAD, thereby rendering it unable to antagonize the anti-apoptotic protein BCL-xL (266). Additional survival mechanisms include RSK-mediated phosphorylation and inactivation of the death-associated protein kinase (DAPK) (267), RSK1-mediated phosphorylation of the CCAAT/enhancer binding protein β (C/EBP β) (268), RSK2-mediated phosphorylation of CREB at Ser133 to increase transcription of pro-survival proteins and factors (e.g. BDNF) (269), and RSK1-mediated degradation of the NFκB inhibitor IkBα (255).

In addition to the cellular processes discussed above, RSKs regulate a plethora of ‘other’ processes. For instance RSK1-mediated phosphorylation regulates neuronal nitric oxide synthase (NOS) activity (270) and promotes neurite outgrowth and differentiation of PC12 cells (271). RSK2, as well, regulates additional processes including neurite outgrowth via phosphorylation of the cell adhesion molecule L1 (272) and mitogen-
dependent Na+/H+ exchange and intracellular pH via NHE1 phosphorylation (273). Significantly, RSK2 is a crucial signaling effector in FGFR3 expressing myeloma cells (239); whereas RSK3 functions as a potential tumor suppressor in ovarian cancer (274) and RSK4 participates in p53-dependent cell growth arrest (275). Additional evidence for RSK activity in cancer comes from inhibitor studies using the RSK-specific NTD inhibitor SL0101 (276). It was found that inhibition of RSK selectively inhibits proliferation of MCF-7 breast cancer cells, as well as LNCaP and PC-3 prostate cancer cell lines (277). It is clear from these studies that RSKs mediate an enormous variety of signal transduction events and underscores the importance of these kinases in proper cellular function.

1.3.7.4.3 RSK and Human Disease: Role of RSK2 in Coffin-Lowry Syndrome

To date, 128 mutations have been identified in the human gene encoding RSK2 (RPS6KA3, XP22.2) (278), which is clearly associated with the X-linked Coffin-Lowry Syndrome (236,279). First reported in the clinic by Coffin et al. (280) and Lowry et al. (281), and later classified as a distinct syndrome (282), Coffin-Lowry Syndrome is characterized by psychomotor retardation, hypotonia (i.e. decreased muscular tone), and characteristic facial, hand, and skeletal abnormalities (283). Moreover, cognitive function is moderately impaired (i.e. IQ usually <55) and is not as severe as previously reported due to better early intervention methods (284). Significantly, Coffin-Lowry Syndrome has been associated with episodic or long-standing psychotic behavior in females from four different families, wherein three were diagnosed as having either schizophrenia or depressive psychosis (283). Importantly, these symptoms are consistent with reports of
impaired learning and cognition, poor coordination, and progressive skeletal disease in RSK2 knock-out mice (246,285), as well as impaired operant and Pavlovian (classical) learning in *Drosophila melanogaster* expressing RSK2 null mutations (286).

Of the 128 known mutations, 33% are missense, 15% nonsense, 20% splicing errors, and 32% represent deletions or short insertion events, with the majority of mutations occurring within the NTD (53%) or the CTD (33%) (278). It is predicted that at least 66% of these mutations result in premature translation termination, thereby causing a complete loss of RSK2 activity. Many of the missense mutations alter known phosphorylation sites critical for RSK2 catalytic function, ATP binding, or ERK binding (283). Considering the overwhelming evidence showing that RSK2 signaling is important for various cellular functions, it is no surprise that these mutations affecting RSK2 activation lead to human disease.

### 1.3.8 RSK2 Interacts with the 5-HT$_{2A}$ Receptor and Negatively Regulates Signaling

It was recently discovered, via a yeast-two-hybrid screen using the human 5-HT$_{2A}$ receptor i3 loop as ‘bait’ and a human cDNA library as ‘prey’, that RSK2 interacts with the 5-HT$_{2A}$ receptor within a region of the i3 loop that contains an RSK2-like consensus phosphorylation motif ($^{275}$R-A-K-L-A-S$^{280}$) (172). Specifically, Sheffler et al. (172) showed that RSK2 interacts and localizes with 5-HT$_{2A}$ receptors *in vitro* (i.e. HEK293 and C6 glioma) and *in vivo* (rat brain). Immunohistochemical studies in rat brain showed robust colocalization in both the globus pallidus and prefrontal cortex, two regions of the rat brain enriched in 5-HT$_{2A}$ receptors. Significantly, RSK2 extensively colocalized with
5-HT$_{2A}$ receptors within layer V cortical pyramidal neurons, an intriguing finding considering the hypothesis that aberrant 5-HT$_{2A}$ signaling in pyramidal neurons underlies psychotic behavioral states (139).

It was clear then that RSK2 interacts with the 5-HT$_{2A}$ receptor and is present at the same subcellular locations. Therefore it remained to be determined whether or not the RSK2 interaction had functional consequences. Using mouse embryonic fibroblasts (MEFs) isolated from wild type (RSK2+/+) and RSK2 knock-out (RSK2-/-) mice (252), Sheffler and colleagues (172) showed that RSK2 negatively regulated multiple aspects of 5-HT$_{2A}$ signaling as evidenced by significant potentiations in 5-HT-induced IP accumulation, Ca$^{2+}$ release, and ERK1/2 phosphorylation in RSK2-/- mice. Moreover, RSK2 was found to globally regulate GPCR signaling since the signaling of additional GPCRs (i.e. P2Y-purinergic, PAR-1 thrombinergic, β$_1$AR, and bradykinin-B receptors) was similarly potentiated in RSK2-/- MEFs. Recent evidence suggests that kinases can regulate GPCRs in a phosphorylation-independent manner, for example by interacting with or even directly phosphorylating G proteins to inhibit their signaling (287,288). Therefore, it was a distinct possibility that RSK2 modulates 5-HT$_{2A}$ signaling downstream of the receptor. To address this, Sheffler et al. (172) conducted a battery of control experiments showing that RSK2 modulated 5-HT$_{2A}$ receptor signaling independent of changes in 5-HT$_{2A}$ receptor sub-cellular distribution, global G protein function, and without altering the expression of any genes known to be involved in serotonergic signal transduction. These findings were extremely important since they showed that RSK2 acts proximal to receptor activation, at the level of receptor-G protein coupling, perhaps via direct phosphorylation of 5-HT$_{2A}$ receptors. This interpretation
seemed plausible considering that RSK2 interacts with the 5-HT$_{2A}$ receptor within a region harboring an RSK2-like consensus phosphorylation motif. The exhaustive analysis of the mechanism responsible for RSK2-mediated regulation of the 5-HT$_{2A}$ receptor is the main focus of this thesis and will be detailed in Chapter 3. In addition, the experiments in Chapter 3 led to the discovery of two novel mechanisms of receptor regulation which will be detailed in Chapters 4 and 5.
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 General Reagents

5-hydroxytryptamine creatinine sulfate (Serotonin, 5-HT), (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI), quipazine, 5-methoxy-N,N-dimethyltryptamine (5-methoxyDMT), 1-(m-chlorophenyl)-piperazine (m-CPP), R(+)SCH-23390, α-methyl serotonin (α-methyl5-HT), M2 FLAG affinity resin, FLAG peptide, rabbit polyclonal anti-FLAG antibody, human EGF, human PDGF, human TGF-α, papain, LiCl, probenecid, porcine gelatin, paraformaldehyde, Triton X100 and all other standard reagents were supplied by Sigma-Aldrich Corp. (St. Louis, MO). MK212 was obtained from Tocris Bioscience (Ellisville, MO). MDL100907 and lisuride were acquired as previously detailed (145). N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was supplied by Acros Organics (Morris Plains, NJ). SL0101 was supplied by Toronto Research Chemicals, Inc. (Ontario, Canada) and BI-D1870 was supplied by the Division of Signal Transduction Therapy, University of Dundee (Scotland, UK). Collagenase II was obtained from Worthington Biochemical Corp. (Lakewood, NJ) and elastase (grade II) was supplied by Roche Applied Science (Indianapolis, IN). Hoechst, Concanavalin A-Alexa Fluor 488nm, and primers for molecular biology were supplied by the Invitrogen Corp. (Carlsbad, CA). Restriction endonucleases were supplied by New England Biolabs (Ipswich, MA). [3H]-ketanserin (67.0 Ci/mmol), [3H]-myo inositol (21.7 Ci/mmol), [γ-32P]-ATP (3000 Ci/mmol), and [32P]i (orthophosphate, 285.6 Ci/mg) were obtained from
PerkinElmer Life and Analytical Sciences (Waltham, MA). Purified, activated RSK2 was purchased from Upstate-Millipore (Billerica, MA).

2.1.2 Cell Culture Reagents

2.1.2.1 General

Cell culture reagents including fetal bovine serum (FBS), Dulbecco’s modified essential medium (DMEM), Trypsin-EDTA, F-12 nutrient mixture, OptiMEM, neurobasal medium, freezing medium, sodium pyruvate, penicillin, and streptomycin were supplied by Gibco (Invitrogen, Carlsbad, CA). Inositol-free basal medium Eagle’s (BME) was obtained from Cambrex (East Rutherford, NJ). Human Embryonic Kidney 293T (HEK 293T) cells were purchased from the American Type Culture Collection (Manassas, VA) and the RSK2+/+ and RSK2-/- mouse embryonic fibroblasts (MEFs) used for generation of stable lines were isolated according to Bruning et al. (252). Mouse aortic vascular smooth muscle cells (mVSMCs) and cortical neurons were isolated from RSK2+/+ and RSK2-/- mice as detailed below.

2.1.2.2 Serum Dialysis

Extensive dialysis of FBS was performed to remove 5-HT present in the serum. I found that this method yielded superior results compared to a commercial source. Briefly, 500 mL of FBS was thawed and approximately 40 mL was placed inside 22 inch sections of washed dialysis tubing (Spectra/Por 3500 MWCO, Spectrum Laboratories, Rancho Dominguez, CA). Dialysis tubes were suspended in a 4 L graduated cylinder containing 4 L of cold dialysis buffer (120 mM NaCl, 10 mM Tris-HCl, pH 7.5 at RT) and
equilibrated for 24 hr at 4°C with stirring. The buffer was changed five times for a total of 120 hr of dialysis. After the final equilibration, dialyzed FBS was sterile-filtered (Millipore 0.22 µm filter unit), aliquoted, and frozen at -20°C. Dr. Betsy Pehek analyzed the dialyzed serum against the undialyzed Gibco-FBS and dialyzed Gibco-FBS via high performance liquid chromatography-electrochemical detection (HPLC-ECD) and found that our dialyzed serum contained 0.77 nM 5-HT, whereas commercial dialyzed FBS contained 26 nM 5-HT, and untreated FBS contained 470 nM 5-HT (Figure 2-1).
Figure 2-1. High performance liquid chromatography-electrochemical detection (HPLC-ECD) analysis of ‘Roth-dialyzed’ fetal bovine serum. Fetal bovine serum (FBS) was extensively dialyzed and 20 µL aliquots were analyzed via HPLC-ECD by Dr. Betsy Pehek. The ‘Roth-dialyzed’ FBS (A) was then compared to 20 µL aliquots of Gibco-dialyzed FBS (B) and untreated FBS (C). A, Each 20 µL aliquot of ‘Roth-dialyzed’ FBS contained 6.0 pg 5-HT. B-C, By comparison, the commercial dialyzed
serum contained 205 pg 5-HT and the untreated serum contained 3610 pg 5-HT. These data indicate that the ‘Roth-dialyzed’ serum is superior to the commercial dialyzed serum and allows us to culture cells in medium containing 39 pM 5-HT, as opposed to 1.3 nM 5-HT (i.e. medium with 5% serum).

2.1.3 cDNA Constructs

2.1.3.1 Insertion of the Rat 5-HT2A Receptor into pBABEpuromycin for Retrovirus Production

The wild-type rat 5-HT2A receptor containing a cleavable N-terminal H. influenzae hemagglutinin membrane insertion signal sequence (289) and N-terminal FLAG (DYKDDDDK) affinity tag (i.e. the rat Signal-FLAG 5-HT2A receptor construct, referred to here as FLAG 5-HT2A) was constructed previously (209). Importantly, the FLAG 5-HT2A construct, as it is named in several papers (172,209,290), should not be confused with the FLAG-tagged 5-HT2A receptor in pCMV TAG2b which lacks the signal sequence and therefore has low expression. All experiments conducted in this thesis incorporated the 5-HT2A receptor with the N-terminal signal sequence. As shown in Figure 2-2, 5’ EcoRI and 3’ Sall restriction sites were introduced into the FLAG 5-HT2A sequence via the following PCR primers: 5’AAAGAATTCGCCACCATGAAGACGATCAT3’ (EcoRI highlighted) and 5’AAAGGTGCAGTCACACACAGCTAACCTTTTC3’ (Sall highlighted). The amplicon was then subcloned into the pBABE retroviral vector containing a puromycin resistance gene (pBABEpuro) (291) and sequence-confirmed (Case Genomics Core Facility, Cleveland, OH). Although not used in this thesis work, both the wild-type and FLAG-
tagged (lacking signal sequence) rat 5-HT$_2$A receptors were subcloned into pBABEpuro via this approach and will be included here for completeness. Specifically, the following primers were used: 5’AAAGAATTCGCCACCATGGAAATTCTTTTGTGAAGAC3’ and 5’AAAGTCGACTCACAACAGCTAACCCTTTC3’ for wild-type receptor (originally in pCMV TAG2B), and 5’AAAGAATTCGCCACCATGGAATTACAAGGATGACGAC3’ and 5’AAAGTCGACTCACAACAGCTAACCCTTTC3’ for FLAG-tagged receptor (originally in pCMV TAG2B). All constructs expressed native 5-HT$_2$A receptors as determined via [³H]-ketanserin competition binding experiments (Figure 2-3). Both the antagonist chlorpromazine (one-site binding curve, K$_i$~1 nM) and endogenous full agonist 5-HT (two-site binding curve, K$_{i1}$~5-11 nM and K$_{i2}$~400-600 nM) bound with characteristic affinities (see Chapter 2.2.6).
FLAG 5-HT<sub>2A</sub> pBABEpuro construct:

**EcoRI** - **Kozak** - **H. influenzae** hemagglutinin cleavable membrane insertion signal sequence - **FLAG epitope tag** - **Linker** - Rat 5-HT<sub>2A** - Stop - **Sall**

cDNA (sequence confirmed):

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GAATTCGGCCACATGAAAGACGATCATGGCCCTAGAGCATCTCCATCTGCTGGTATTCGCGGATACAAGAAGCTAGCGAG
```

Figure 2-2. Insertion of the wild-type rat FLAG-tagged 5-HT<sub>2A</sub> receptor with a cleavable N-terminal membrane insertion signal peptide into the pBABEpuro retroviral vector. The wild-type rat 5-HT<sub>2A</sub> receptor construct containing both a cleavable *H. influenzae* hemagglutinin membrane insertion signal sequence...
(MKTIIALSYIFCLVFA) and a FLAG affinity tag (DYKDDDDK) in the amino-terminus (FLAG 5-HT$_{2A}$) was constructed previously (213). This construct was PCR amplified and subcloned into the pBABEpuro retroviral vector via EcoRI and SalI restriction sites, as shown here. The same approach was used to insert the wild-type and FLAG-tagged rat 5-HT$_{2A}$ receptors (both lacking the signal sequence) into pBABEpuro. All full-length constructs were sequence-verified.

Figure 2-3. Validation of tagged and un-tagged rat 5-HT$_{2A}$ receptors cloned into the pBABEpuro retroviral vector via competition radioligand binding experiments. Competition radioligand binding experiments were performed using 0.5 nM [³H]-

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ketanserin, chlorpromazine or 5-HT as competing ligands, and membranes isolated from HEK293T cells transfected with pBABEpuro plasmids containing Signal-FLAG (FLAG 5-HT_{2A}) (A), FLAG (no signal sequence) (B), and wild-type (C) rat 5-HT_{2A} cDNA. Non-specific binding was determined with 10 µM chlorpromazine. Equilibrium inhibition constants (K\text{\textsubscript{i}} values) were determined via nonlinear regression using a K\text{\textsubscript{d}} of 0.8 nM and agreed with published reports (http://pdsp.med.unc.edu/indexR.html).

2.1.3.2 Insertion of the Rat 5-HT\textsubscript{2A}-GFP-CT Receptor into FUGW for Lentiviral Production

Lentiviruses containing fluorescent receptor constructs with 5’ Kozak sequences were cloned by PCR amplification and ligation. The 5-HT\textsubscript{2A}-GFP-CT sequence (213) was PCR amplified and ligated into the lentiviral construct FUGW (292) using the following primers: AAAA[TCTAGA]GCCACCATGGAAATTCTTTGTGAAG (XbaI highlighted) and TTTT[GAATTCT]CACACACAGCTAACCTTTTCATTTC (EcoRI highlighted). The resulting FUGW construct was sequence verified by automated sequencing at the UNC-Chapel Hill DNA sequencing facility. For viral production, the FUGW receptor construct was co-transfected along with viral packaging constructs VSVG and Delta 8.9 (ratio= 3.3 FUGW: 2.5 Delta 8.9 : 1 VSVG) into seven 150 cm\textsuperscript{2} dishes of HEK2T cells using a 5:1 ratio of Fugene:DNA. Forty-eight hours after co-transfection the media containing virus was removed and viral pellets were obtained by centrifugation (5 hr at 26,000 x g). The virus was resuspended in PBS and then concentrated from 12 ml to 200 µl using Amicon Ultra filters (Millipore). The resulting
concentrated lentivirus was used to infect cultures of primary cortical neurons. This work was carried out by Dr. John Allen and included here for completeness.

2.1.3.3 Construction of a Tandem Affinity-Tagged 5-HT$_{2A}$ Receptor for Receptor Purification

The wild-type rat 5-HT$_{2A}$ receptor containing an N-terminal *H. influenza* hemagglutinin membrane insertion signal sequence and contiguous FLAG and hexahistidine affinity tags (FLAG-His$_6$ 5-HT$_{2A}$) was constructed using the rat FLAG 5-HT$_{2A}$ receptor (213) as a template for overlap extension PCR (Figure 2-4). Briefly, two amplicons inserting 5′ EcoRI (amplicon 1) and 3′ SalI (amplicon 2) restriction sites as well as an overlapping hexahistidine affinity tag sequence were generated. Amplicon 1 was generated with the following primers: AAAAGAATTCCGCCACCATGAAGACGATCAT (EcoRI highlighted) and GATGTGATGATGATGATGATGCTTATCGTCGTTCATCCTTG (Hexahistidine sequence highlighted). Amplicon 2 was generated with the following primers: CATCATCATCATCATCACATCGAGGGCCCG (Hexahistidine sequence highlighted) and AAAGTCTAGCTCACACACAGCTAACCTTTTCATTC (SalI highlighted). The amplicons were annealed and extended to produce the full-length FLAG-His$_6$ 5-HT$_{2A}$ product. The FLAG-His$_6$ 5-HT$_{2A}$ cDNA was subcloned into the pBABEpuro retroviral vector for stable line generation using the EcoRI and SalI amplicon primers. The phosphorylation-deficient mutant (S314A) in pBABEpuro was mutagenized as outlined below. The FLAG-His$_6$ 5-HT$_{2A}$ receptor insert in pBABEpuro was subcloned into pcDNA3 for receptor purification using the following primers:
AAAAAGCTTACCACCATGAAGAC (HindIII highlighted) and AAATCTAGATCACACAGCTAACCTTTTCATT (XbaI highlighted). Each FLAG-His$_6$ 5-HT$_2$A receptor construct was confirmed by automated sequencing (UNC-CH Genome Analysis Facility, Chapel Hill, NC) and alignment of the overlapping sequences in Vector NTi (Invitrogen Corp.). Importantly, the addition of N-terminal affinity tags did not perturb overall receptor conformation since the affinity tagged receptor bound [$^3$H]-ketanserin with a $K_d$ equal to 2.65 +/- 0.39 nM (N=4), consistent with reported affinities for the 5-HT$_2$A receptor (http://pdsp.med.unc.edu/pdsp.php).
Figure 2-4. Construction of a Tandem Affinity-Tagged 5-HT$_2A$ Receptor for Receptor Purification. The 5-HT$_2A$ receptor containing a cleavable H. influenzae hemagglutinin membrane insertion signal sequence (MKTIIALSYIFCLVFA) and...
contiguous FLAG (DYKDDDDK) and hexahistidine (His$_6$) affinity tags in the amino terminus was constructed via overlap extension PCR. This construct was PCR amplified and subcloned into the pBABEpuro retroviral vector via EcoRI and SalI restriction sites, and subsequently into pcDNA3 via HindIII and XbaI restriction sites. The full-length receptor was sequence-verified and shown to bind ketanserin with characteristic affinity.

2.1.3.4 Construction of a Tandem Affinity-Tagged I3 Loop Peptide Construct for Purification

The tandem affinity-tagged i3 loop peptide fusion construct (amino acids 252-328 of the rat 5-HT$_{2A}$ receptor) was detailed previously (199) but will be described here briefly to address some methodological inconsistencies. This cloning and purification strategy yielded a 79 amino acid peptide (theoretical MW = 8772 Da, apparent MW <6000 Da) containing an extra N-terminal Met and extra C-terminal Gly. As shown in Figure 2-5, 5’ NdeI and 3’ SmaI sites were introduced into the i3 loop sequence via the following PCR primers: ACCATCCATATGATCACCTACTTCTGACTATCAAG (NdeI highlighted) and AAAACCAGGCACGATGCCCAGCACCTTGACGCCCTTT (SmaI highlighted). The amplicon was purified, digested, and subcloned into the pCYB2 vector (IMPACT, New England Biolabs, Inc.). The resulting construct was subcloned into the inducible pET11a expression vector (Novagen, Madison, WI) using the NdeI forward primer (above) and a NheI reverse primer containing a hexahistidine affinity tag: 5’

TATGCTAGCTTAGTGGTGCTGTTGTTGTTGTTTGAAGCTGCCACCAAGGCAG
G3’ (NheI highlighted and hexahistidine underlined). The outcome was an IPTG-inducible i3 loop fusion protein located N-terminal to a self-splicing element (intein) and two affinity tags (i.e. chitin binding domain engineered to contain a C-terminal hexahistidine affinity tag, MW~60 kDa) (Figure 2-6). The i3-inteinCBD-His\textsubscript{6} construct was sequence-verified (Case Genomics Core Facility, Cleveland, OH) and validated for expression in \textit{E.coli} (Figures 2-5 and 2-6). Phosphorylation-deficient mutants were constructed as outlined below.
Figure 2-5. Construction of the i3-inteinCBD-His$_6$ fusion protein for tandem affinity purification of rat 5-HT$_{2A}$ i3 loop peptide. The rat 5-HT$_{2A}$ receptor third intracellular loop (i3) was cloned into the amino-terminus of a fusion protein.
containing a self-splicing element (intein) and two affinity tags (i.e. chitin binding domain engineered to contain a C-terminal hexahistidine affinity tag, MW~60 kDa). This fusion protein was used to generate a 79 amino acid peptide incorporating amino acids 252-328 of the rat 5-HT$_{2A}$ receptor.

Figure 2-6. Schematic showing tandem affinity purification of the rat 5-HT$_{2A}$ i3 loop peptide using the i3-inteinCBD-His$_6$ fusion protein. A, The rat 5-HT$_{2A}$ receptor third intracellular loop (i3) was purified from an i3-inteinCBD-His$_6$ fusion protein.
immobilized on Ni$^{2+}$-NTA and chitin resins. The peptide was liberated via an intein self-cleavage event under reducing conditions. B, Induction of OD$_{600}$ nm $E. coli$ cultures yielded a soluble i3-inteinCBD-His$_6$ fusion protein (apparent MW~60 kDa). C, Western blot showing successful i3 loop peptide purification from $E. coli$ as an i3-inteinCBD-His$_6$ fusion protein followed by intein self-cleavage and filtration and concentration steps. According to our previous work (199), this scheme yields i3 loop peptides that adopt a predominantly alpha-helical secondary structure and are capable of binding purified arrestins.

2.1.3.5 Construction of GFP-Tagged RSK2

The native mouse RSK2 cDNA was subcloned from pMT2-HA-RSK2 (293) into pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA) by introducing 5’HindIII and 3’KpnI sites using the following primers:

AAAAAAGCTTTAGCCACCATGCCTGGCGCAGCTG (HindIII highlighted) and ACCTCAACAGCCCTGTGAGGTACCTTTT (KpnI highlighted). Kinase-dead (Lys to Ala mutation in the conserved ATP binding site) RSK2 mutants in pEGFP-C1 were generated as outlined below and sequence-verified (Case Genomics Core Facility, Cleveland, OH). This work was carried out by Dr. Douglas J. Sheffler and included here for completeness.
2.1.4 Antibodies

2.1.4.1 Commercial Antibodies

The polyclonal rabbit GFP antibody (Ab) was obtained from Abcam (Cambridge, UK). The polyclonal rabbit phospho-p90RSK antibody (Ser(P)-386 Ab) was from Cell Signaling Technology, Inc. (Danvers, MA). The rabbit polyclonal Gαq, goat polyclonal and mouse monoclonal anti-RSK2 antibodies, and Protein A/G PLUS beads were from Santa Cruz Biotechnology (Santa Cruz, CA). The HRP-conjugated goat anti-rabbit and horse anti-goat secondary antibodies were from Vector Laboratories (Burlingame, CA). Alexa Fluor 594 nm goat anti-rabbit secondary antibody was supplied by Invitrogen Corp. (Carlsbad, CA).

2.1.4.2 Custom Antibodies

Generation of a rabbit polyclonal antibody targeting amino acids 280–295 of the rat 5-HT2A receptor (antibody 1A), displaying a titer of 1:30,000 against the synthetic peptide and at least 1:20,000 against the purified i3 peptide, was described previously (199).

Rat 5-HT2A receptors were detected in Western blots with an antibody targeting amino acids 428–443 (428QKKNSQEDAEQTVDDC443) of the rat 5-HT2A C-terminus (5-HT2A-CT Ab). This antibody was generated as described previously (294), and as shown in Figure 2-7, raw serum from animal #9753 tested positive for reactivity with purified 5-HT2A receptors. Specifically, serum from the ten-week and extension bleeds (extensions 1A, 1B, and 1C) detected purified rat 5-HT2A receptors, but not purified 5-HT2C receptors, and this recognition was blocked by the antigenic peptide (Figure 2-7).
However, its performance in immunofluorescence assays has not been rigorously evaluated.

An antibody was developed to detect phosphorylation of rat 5-HT$_{2A}$ receptors at Ser280 (Ser(P)-280) by targeting amino acids 270–284 within the 5-HT$_{2A}$ receptor i3 loop. Briefly, two New Zealand white rabbits were injected with a synthetic peptide ($^{270}$SDLSTRAKLA-S(PO$_3$)-FSFL$_{284}$-Cys) conjugated to keyhole limpet hemocyanin in Freund’s complete adjuvant (Biomer Technology, Hayward, CA). Following a standard immunization, boost, and bleed schedule, raw serum was characterized via dot blots using both the unmodified peptide and the phosphopeptide. Antibodies in unpurified raw serum from animals 143 and 144 preferred the phosphopeptide at high dilutions (Figure 2-8A). All experiments were performed using the third bleed from animal #144 which can selectively detect ~1 ng of the phosphopeptide.

Phosphorylation of rat 5-HT$_{2A}$ receptors at Ser314 (Ser(P)-314) was detected by targeting amino acids 307–322 within the C-terminus of the 5-HT$_{2A}$ receptor i3 loop. Briefly, two New Zealand white rabbits were injected with a synthetic peptide ($^{307}$AGRRTMQ-S(PO$_3$)-ISNEQKAC$_{322}$) conjugated to keyhole limpet hemocyanin in Freund’s complete adjuvant (Biomer Technology, Hayward, CA). Subsequent to a standard immunization, boost, and bleed schedule, bleeds were characterized via dot blots using both the unmodified peptide and the phosphopeptide. Antibodies in raw serum from animals 157 and 158 preferentially detected the phosphopeptide at high dilutions (Figure 2-8B). All experiments were performed using the second bleed from animal #157 which can selectively detect <10 ng of the phosphopeptide.
Figure 2-7. Characterization of the 5-HT\textsubscript{2A} receptor C-terminal antibody. Raw serum from rabbit #9753 was capable of detecting purified rat 5-HT\textsubscript{2A} receptors via Western blotting. A, Raw serum from the ten week bleed differentiated between purified rat 5-HT\textsubscript{2A} and purified rat 5-HT\textsubscript{2C} receptors. Notice the lack of immunoreactivity in the pre-immune serum and the ability for the antigenic peptide
to block 5-HT\textsubscript{2A} detection. B, Raw serum from extension bleeds (i.e. extensions 1A, 1B, and 1C) also differentiated between purified rat 5-HT\textsubscript{2A} and 5-HT\textsubscript{2C} receptors. Moreover, this recognition was not present in the pre-immune serum and was blocked by the antigenic peptide. The inconsistencies in 5-HT\textsubscript{2A} receptor mobility in panel B are most likely due to differences in glycosylation since receptors were purified from two different cell lines (i.e. NIH3T3 and HEK293 cells).

Figure 2-8. Characterization of Ser(P)-280 and Ser(P)-314 phospho-specific antibodies. A, Dot blot analysis of raw serum obtained from animals #143 and #144 shows that both animal sera contain antibodies specific for the Ser280 phosphopeptide over the unphosphorylated peptide and detect <10 ng of phosphopeptide at a dilution of 1:1000. Immunoreactivity was absent from both pre-immune sera. B, Dot blot analysis of raw serum obtained from animals #157 and #158 shows that both animal sera contain antibodies that differentiate between
the Ser314 phosphopeptide and the unphosphorylated peptide with a sensitivity of approximately 10 ng of phosphopeptide at a dilution of 1:2000. Immunoreactivity was absent from both pre-immune sera. Bleed 2 from animal #157 was used for all experiments requiring the Ser(P)-314 Ab.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 General Cell Culture

All cell lines were cultured at 37ºC in a humidified environment in the presence of 5% CO₂. HEK 293T, HEK 293TS, mouse embryonic fibroblasts (MEF), and mouse aortic vascular smooth muscle (mVSMCs) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. MEFs that were stably transduced with virus were cultured in the presence of 4 μg/mL puromycin to maintain selection pressure. Cells were cryopreserved using freezing medium and transferred to liquid nitrogen following initial freezing at -80ºC.

2.2.1.2 Isolation of Mouse Aortic Vascular Smooth Muscle Cells

Mouse aortic vascular smooth muscle cells (mVSMCs) were isolated from 12-week old mice (three mice per genotype). Briefly, each mouse was sacrificed by cervical dislocation and cleaned with 70% ethanol. Under sterile conditions the mouse was perfused with 25 mL of 1X HBSS (Gibco #14175, without Ca²⁺ and Mg²⁺) and the abdominal/thoracic aorta extending from the ilial bifurcation to aortic arch was
microdissected. The dissected aorta were placed in RT HBSS and quickly cleaned of any residual fat. The aorta were placed in a fresh 6 cm dish containing 6 mL of collagenase buffer (175 U/mL in HBSS, filtered through 0.2 µm polyethersulfone membrane) and incubated for 15 min at 37°C in the presence of 5% CO₂. The aorta were transferred to a fresh 6 cm dish containing HBSS and the adventitia was removed. The cleaned aorta were then placed in DMEM supplemented with 10% FBS and incubated overnight at 37°C in the presence of 5% CO₂. The next day the pooled aorta were cut into 2 mm segments and transferred to a fresh 6 cm dish containing 6 mL of digestion buffer (175 U/mL of collagenase and 0.125 mg/mL of elastase in HBSS, filtered through 0.2 µm polyethersulfone membrane). The aorta were digested for 60 min at 37°C in the presence of 5% CO₂, followed by extensive trituration with a glass Pasteur pipette. When completely dissociated, 10 mL of DMEM supplemented with 10% FBS was added to the digest and the cells were collected via centrifugation at 200 x g for 8 min. The cell pellet was resuspended in 3 mL DMEM supplemented with 20% FBS and quantitatively transferred to a T-25 cm flask (normal tissue culture treated) with an additional 2 mL of medium. The cells were incubated overnight at 37°C in the presence of 5% CO₂. The next day the cells were carefully washed with DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 µg/mL gentamycin. The cells were then cultured normally with medium changes every three days.

2.2.1.3 Primary Cortical Neuron Culture and Lentiviral Infections

Cortical neurons were prepared as previously described by others (295). Briefly, newly born animals (postnatal day ≤ 1 day) were collected, genotyped and euthanized by
decapitation. The entire frontal cortex was dissected from whole brain, followed by digestion in neurobasal medium (containing 0.1% Papain and 0.2% BSA) at 37°C for 20 min. Papain was removed and replaced with complete medium (neurobasal medium, 1x B27 supplement, 10 mg/mL L-glutamine, and 10 mg/mL glutamate) and digested tissue was mechanically broken via trituration with Pasteur pipettes. The supernatant was transferred to a new sterile 1.5 mL tube, leaving the aggregates, and spun down at 200 x g for 10 min. The supernatant was discarded and the pellet resuspended in pre-equilibrated (to 37°C and 5% CO₂) neurobasal medium containing B27 supplement, antibiotics, and 0.5 mM glutamine. Cells were counted and seeded onto poly-L-lysine-coated 96 well plates (poly-L-lysine solution: 0.1 mg/ml low molecular weight poly-L-lysine, 0.625% boric acid, 0.955% Borax). Cells were seeded into 96 well plates at a density of 5 x 10⁴ cells/well and maintained in 5% CO₂ at 37°C. The primary cortical neuron cultures were grown 7 to 10 DIV followed by viral infection (10 µl/25 mm dish or 1.5 µl/96 well plate) using lentiviruses containing 5-HT₂A-GFP-CT.

2.2.2 Transfection

Fugene6 (Roche) was used exactly as described by the manufacturer to transiently transfect subconfluent HEK 293T cells cultured in 10 cm dishes. Briefly, Fugene was incubated with 600 µL of F-12 medium for 5 min at RT, followed by addition of ethanol-precipitated plasmid DNA (5:1 ratio of Fugene:DNA). Fugene-DNA complexes were allowed to form over 45 min at RT and then added to the cells. Large transfections were pooled with no loss of transfection efficiency. Transfected cells were used within 72 hr of transfection.
2.2.3 Retrovirus Production and 5-HT$_{2A}$ Polyclonal Stable Line Generation

Amphotropic retrovirus was produced from helper virus-free HEK 293 (HEK 293TS) host cells using the pBABEpuro retroviral vector and pCL10A1 amphotropic packaging plasmid (Imgenex, San Diego, CA). Briefly, HEK 293TS cells were co-transfected with 3 μg of cDNA (e.g. RSK2- or 5-HT$_{2A}$-containing plasmids) and 3 μg pCL10A1. Viral supernatants were collected 24-36 hr post-transfection, sterile filtered through a 0.45 μm Acrodisk (HT tufftyn membrane), and immediately frozen on dry ice. Viral supernatants were stored at -80°C until further use and were never thawed more than once.

For stable line generation, viral supernatants were quickly thawed at 37°C, combined with polybrene (8 μg/mL), and added to subconfluent RSK2+/+ and RSK2-/- MEFs. Polyclonal cell populations were selected with puromycin (3-4 μg/mL) 48 hr after infection, as determined from kill curve experiments. Polyclonal populations of fibroblasts expressing wild-type and 5-HT$_{2A}$-S314A receptors were expanded and characterized for receptor expression using radioligand binding as described below.

2.2.4 Site-Directed Mutagenesis

The QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate kinase-dead RSK2 mutants (courtesy of Douglas J. Sheffler and included here for completeness), i3 loop peptide mutants, and a full-length FLAG-His$_6$ 5-HT$_{2A}$ receptor mutant using the wild-type constructs as PCR templates. The following primers were used to generate kinase-dead RSK2 mutants (mutated codon highlighted):
1) K100A- GCTAGACAGCTTTATGCCATGGCAGTATTAAGAAGGCCAC and GTGGCCTTCTTTAATACTGCCCATGGCATAAAGCTGTCTAGC, 2) K451A- CAAACATGGAGTTTGCCTGGCATTTGATAAAAAGCAAGAGAG and CTCTCTTGCTTTTATCAATAATCGCCACGGCAAACCTCCATGTITTG. The S280A i3 loop peptide mutant in pET11a was generated using mutagenesis primers ACTCGAGCCAAACTAGCCGCCCTTCAGCTTCTCCTCCTCAG and CTGAGGGAGGAAGCTGAA GGCGGTAGTTTGGCTCGAGT. The phosphorylation-deficient S314A i3 loop peptide and receptor mutants were both generated using the mutagenesis primers GGCCGAAGGACGATGCAG GCCATCAGCAATGAGCAAAAG and CTTTGCTCATGCTGATGGCCCTGCATCGTCCTCGGCCC. The full-length constructs were sequence-verified (Genomics Core Facility, Cleveland, OH and UNC-CH Genome Analysis Facility, Chapel Hill, NC) and aligned in Vector NTi (Invitrogen).

2.2.5 Tandem Affinity Purification of 5-HT2A Receptors and I3 Loop Peptides

2.2.5.1 5-HT2A Receptor Purification

Tandem affinity-tagged rat 5-HT2A receptors were purified to near homogeneity for in vitro kinase assays. Each 10 cm dish of HEK293T cells was transfected with 10 μg FLAG-His$_6$ 5-HT2A cDNA and harvested 48 hr post-transfection in cold PBS (20-10 cm dishes for each round of purification). The cell pellets were solubilized with cold lysis buffer A (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1.0% CHAPS, and EDTA-free protease inhibitor cocktail (Roche), pH 7.5) at a concentration of 10 mL/g wet cell pellet for 20 min at 4°C. The cleared supernatant was added to EZ-view M2 FLAG affinity
resin and rocked for 2 hr at 4°C. The M2 FLAG resin was washed twice with 10 volumes of lysis buffer A and once with 10 volumes of lysis buffer devoid of EDTA (lysis buffer B: 50 mM HEPES, 150 mM NaCl, 1.0% CHAPS, EDTA-free protease inhibitors, pH 7.5). The receptors were eluted from the M2 FLAG resin with 200 μM 1X FLAG peptide, brought to 10 mM imidazole, and added to Ni-NTA resin (Qiagen Inc., Valencia, CA). The Ni-NTA resin was incubated for 2 hr at 4°C with gentle agitation and washed four times with 10 volumes wash buffer (lysis buffer B + 20 mM imidazole, pH 7.5). The FLAG-His$_6$ 5-HT$_2A$ receptor was eluted from the Ni-NTA resin with elution buffer (lysis buffer B + 250 mM imidazole, pH 7.5) and stored at -80°C.

### 2.2.5.2 I3 Loop Peptide Purification

The i3 loop peptide (amino acids 252-328 of the rat 5-HT$_2A$ receptor) was purified similarly to a previous report (199) and yields a 79 amino acid peptide (MW = 8772Da) containing an extra N-terminal Met and extra C-terminal Gly (Figure 2-5 and 2-6). Briefly, BL21(DE3)pLysS E. coli (Stratagene, La Jolla, CA) were freshly transformed with WT and mutant i3 loop peptide constructs in pET11a, grown to $\text{OD}_{600nm}=0.6$ in LB medium containing 60 μg/mL ampicillin and 34 μg/mL chloramphenicol, and induced with 0.5 mM IPTG for 5 hr at 25°C. The E. coli were collected, washed, and frozen at -20°C. The frozen pellets were resuspended in cold lysis buffer (20 mM HEPES, 500 mM NaCl, 0.1% Triton X100, and 100 μM PMSF, pH 8.0), passed three times through a French Press at 1500 psi, and the supernatant was isolated by centrifugation at 12,000 x g for 45 min. The supernatant was loaded onto a Ni-NTA column, washed with 7 bed volumes of wash buffer (20 mM HEPES, 500 mM NaCl, and 20 mM imidazole, pH 8.0),
and the fusion protein was recovered with 3 bed volumes of elution buffer (20 mM HEPES, 500 mM NaCl, and 250 mM imidazole, pH 8.0). Fractions containing the 60 kDa fusion protein, as determined by Western blot analysis, were pooled and applied to a chitin column (New England Biolabs, Inc., Ipswich, MA). The chitin column was washed with 7 bed volumes of wash buffer (20 mM HEPES, 1 M NaCl, pH 8.0) and incubated for 16 hr at 4°C with intein cleavage buffer (20 mM HEPES, 1 M NaCl, 50 mM DTT, pH 8.0). The i3 loop peptide was recovered with 3 bed volumes of elution buffer (20 mM HEPES, 500 mM NaCl, pH 8.0) and each fraction was analyzed by Western blot. Fractions containing the liberated i3 loop peptide (apparent MW=6 kDa) were combined and further purified and concentrated using 30 kDa and 3 kDa Centricon filter units (Millipore, Billerica, MA), respectively. Protein concentrations were determined by the Bradford method (296) and peptides were frozen at -80°C until further use.

2.2.6 Competition Radioligand Binding Assays

Radioligand binding assays were performed with crude cell membranes collected via hypotonic lysis in cold standard binding buffer (50 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.4 at RT) followed by centrifugation at 18,000 x g for 30 min at 4°C (193). Competition assays including 100 µL of drug (competitor) or buffer (total binding) and 50 µL of 2.5 nM [³H]-ketanserin (0.5 nM final) were initiated by adding 100 µL of membrane suspension. Nonspecific binding was defined as radioactivity bound in the presence of 1 µM spiperone or 10 µM chlorpromazine and represented less than 20% of total binding. Assays were incubated for 1 hr at RT and terminated by harvesting (Brandel cell harvester) onto polyethyleneimine-pretreated (0.3%) Whatman GF/C filters.
with extensive washing (three 1 mL ice-cold washes with 50 mM Tris-HCl, pH 7.4 at 4°C). Filters were then soaked in Ecoscint A liquid scintillation cocktail (National Diagnostics; Atlanta, GA) and bound radioactivity was quantified via liquid scintillation counting. Membrane protein concentrations were determined using the Bradford method (296) with BSA as the standard. Heterologous competition binding experiments were analyzed via one- and two-site competition binding curves (Graphpad). Equilibrium inhibition constant values (K_i) were calculated using the equation of Cheng and Prusoff (297). Homologous competition experiments to determine receptor density (B_max) and the equilibrium dissociation constant (K_d) were analyzed via the Ligand program (298).

2.2.7 Immunoprecipitation and Western Blotting

5-HT_{2A} receptors and RSK2 were immunoprecipitated (IP) similarly to previous reports (171,209). In general, cells expressing 5-HT_{2A} receptors, RSK2-GFP, or both were scraped into cold PBS and solubilized with standard lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1.0% CHAPS, protease inhibitor cocktail-EDTA-free, pH 7.5) or phospho-specific lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1.0% CHAPS, protease inhibitor cocktail-EDTA-free, phosphatase inhibitors: 50 mM NaF, 50 mM β-glycerol phosphate, 100 μM sodium orthovanadate, 5 mM sodium pyrophosphate, pH 7.5) for 20 min at 4°C. Supernatants were collected following centrifugation (15,000 x g, 30 min) and equal amounts of lysate were incubated with M2 FLAG beads (5-HT_{2A} IP and RSK2 co-IP) for 2 hr at 4°C with agitation. For detection of phospho-RSK2, equal amounts of Protein A/G cleared lysates were incubated with mouse monoclonal anti-RSK2 for 2 hr at 4°C, followed by an additional incubation with Protein
A/G agarose for 2 hr at 4°C. Immunopurified proteins were extensively washed with lysis buffer, eluted with 2X SDS sample buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 200 mM DTT; 0.2% Bromphenol Blue), and stored at -80°C until further use.

Proteins were immunoblotted using standard procedures (193). Specifically, 5-HT\textsubscript{2A} receptors were resolved on 10% SDS-PAGE gels and i3 loop peptides and *in vitro* phosphorylated 5-HT\textsubscript{2A} receptors destined for mass spectrometry were resolved on 4-20% gradient SDS-PAGE gels (Invitrogen). Proteins were electroblotted onto nitrocellulose membranes (BioRad), and blocked with standard blocking buffer (Tris-buffered saline, 0.1% Tween-20, and 5% nonfat dehydrated milk) or phospho-specific blocking buffer (Tris-buffered saline, 0.1% Tween-20, and 3% bovine serum albumin) for 1 hr at RT. Membranes were washed 3X with Tris-buffered saline + 0.1% Tween-20 (TBST) and the following primary antibodies were used: 5-HT\textsubscript{2A} receptors were detected with the FLAG antibody (1:1000), an antibody directed against the rat 5-HT\textsubscript{2A} C-terminus (5-HT\textsubscript{2A}-CT Ab, 1:1000), or the Ser(P)-314 Ab (1:2000); i3 loop peptides were detected with antibody 1A (1:3000) and the Ser(P)-314 Ab (1:2000); RSK2 was detected using the goat polyclonal RSK2 antibody (1:1000), the rabbit polyclonal GFP Ab, and the rabbit polyclonal Ser(P)-386 Ab (1:1000); total and phosphorylated p42/44 ERK were detected with rabbit polyclonal Thr(P)-202/Tyr(P)-204 and p42/44 ERK (Cell Signaling). Immunoreactive bands were quantified using Kodak Imaging software (Eastman Kodak, New Haven, CT) as discussed below.
2.2.8 In Vitro Kinase Assays

In vitro kinase assays included: 1) purified, activated RSK2 (Upstate-Millipore, Billerica, MA) diluted in enzyme buffer (20 mM MOPS, 1 mM EGTA, 0.01% Brij-35, 5% glycerol, 0.1% β-mercaptoethanol, 1 mg/mL BSA, pH 7.5), 2) i3 loop peptide or FLAG-His₆ 5-HT₂A receptor substrates, and 3) [γ-³²P]-labeled or unlabeled ATP diluted in assay buffer (75 mM MgCl₂, 20 mM MOPS, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, pH 7.2). I assayed for [³²P]-phosphate incorporation by incubating purified i3 loop peptide or purified FLAG-His₆ 5-HT₂A receptor with 0.4-0.8 ng/μL RSK2 in the presence of 0.4-0.04 μCi/μL [γ-³²P]ATP for 1 hr at 30°C. The kinase activity of RSK2 was inhibited in these experiments by using inhibitors of RSK N-terminal kinase domain activity. These inhibitors included SL0101 (10 μM) and BI-D1870 (100 nM) which have been shown to be relatively specific inhibitors of RSK kinase activity (276,299,300). The reactions were terminated by heating the samples for 5 min at 65°C in SDS sample buffer and resolved on 4-20% gradient (Invitrogen) or 10% SDS-PAGE gels. The gels were dried and imaged on a Storm 840 phosphorimager and analyzed using ImageQuant software (Amersham, Buckinghamshire, UK).

Kinetic measurements were performed under initial velocity conditions determined from a pilot experiment showing linear incorporation of [³²P]-phosphate into the i3 loop peptide over 30 min using 0.4 ng/μL RSK2. Briefly, seven concentrations (2.3-140 μM) of i3 loop peptide were incubated with RSK2 in the presence of 10 μM unlabeled ATP and 0.04 μCi/μL [γ-³²P]ATP (7000 cpm/pmol ATP). The reactions were terminated by heating the samples for 5 min at 65°C in SDS sample buffer and resolved.
on a 4-20% gradient SDS-PAGE gel. Radioactivity was quantified by liquid scintillation counting and analyzed by nonlinear regression to determine $V_{\text{max}}$ and $K_{m}$ as discussed below (Graphpad, San Diego, CA). For the mass spectrometry and phospho-specific antibody approaches I incubated the assays for 6 hr at 30°C in the presence of unlabeled ATP (100 μM). Phospho-specific immunoblots were quantified using Kodak Molecular Imaging software (New Haven, CT).

2.2.9 Mass Spectrometry (MS)

MS analyses were performed by Dr. Mike Kinter at the Cleveland Clinic Foundation Lerner Research Institute and Janna Kiselar at the Case Center for Proteomics. Briefly, gel bands containing in vitro phosphorylated i3 loop peptide or 5-HT$_2$A receptor were washed and destained, reduced with DTT, alkylated with iodoacetamide, and digested overnight with an excess of modified trypsin using a standard in-gel digestion protocol. Peptides derived from the i3 loop were extracted from the polyacrylamide and concentrated for LC-tandem MS analysis. The LC-MS system was a ThermoFisher LTQ ion trap mass spectrometer (Thermo Electron Corp., Waltham, MA). Liquid chromatography was performed on an 8 cm x 75 μm id Phenomenex Jupiter C18 reversed-phase capillary chromatography column, packed in-house, coupled to the ThermoFisher LTQ mass spectrometer. Two microliter volumes of the extract were injected, and the peptides eluted from the column by an acetonitrile/0.05 M acetic acid gradient at a flow rate of 0.2 μL/min were introduced into the source of the mass spectrometer on-line. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide
molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans.

Peptides derived from the 5-HT$_{2A}$ receptor were loaded on a TiO$_2$ column (GL Sciences, Inc., Torrance, CA) for enrichment of phosphorylated peptides. Phosphopeptides were eluted with 3% ammonium hydroxide, then concentrated in a vacuum-centrifuge to 2-3 µl and adjusted to a final volume of 5 µl with 1% formic acid/2% acetonitrile in water for LC-MS analysis. Liquid chromatography was performed on a MDLC nano-HPLC (GE Healthcare, Buckinghamshire, UK) coupled to the ThermoFisher LTQ mass spectrometer. The resulting peptides were loaded onto a 300 µm ID x 5 mm C18 PepMap nano Reverse Phase trapping column (Dionex, Sunnyvale, CA) to pre-concentrate and wash away excess salts. The loading flow rate was set to 25 µl/min, with 0.1% formic acid (pH 2.9) as the loading solvent. Reverse phase separation was performed on a 75 µm ID x 15 cm C18, PepMap nano separation column (Dionex, Sunnyvale, CA). Peptide separation was accomplished using buffer A (100% water and 0.1% formic acid) and buffer B (20% water, 80% acetonitrile and 0.1% formic acid). Proteolytic peptide mixtures eluted from the column with the gradient of acetonitrile of 2% per min were introduced into the mass analyzer equipped with a nanospray ion source (2.2 kV).

The i3 loop peptide data were analyzed by a combination of two approaches in order to discover the phosphorylated peptides: 1) all collision-induced dissociation (CID) spectra were used by the search program Sequest (Thermo Electron Corp., Waltham, MA) to search the 5-HT$_{2A}$ i3 loop amino acid sequence considering variable modification of Ser, Thr, and Tyr residues by 80Da, and 2) neutral loss chromatograms were plotted to
facilitate the recognition of CID spectra that contained the characteristic loss of phosphoric acid (H$_3$PO$_4$, -98Da) from phosphoSer- and phosphoThr-containing peptides.

For the 5-HT$_{2A}$ receptor the Mascot search engine (Matrix Science, Boston, MA) was used to search the NCBI Rattus protein database considering Ser, Thr, or Tyr phosphorylation. The first survey MS scan was performed in the positive ion mode. The second LTQ measurement was tandem MS of the four most intense peptide ions and MS-MS-MS (MS$^3$) of all ions showing the neutral loss of H$_3$PO$_4$ (-98 Da) from the precursor ion. All spectra were manually validated and the following acceptance criteria were applied: all phospho-Ser and phospho-Thr peptides were required to show a pronounced neutral loss-dependent MS$^3$ scan and extensive coverage of b- and/or y-type series ions.

2.2.10 $[^{32}\text{P}]$ Metabolic Labeling

The cellular ATP pool was labeled with $[^{32}\text{P}]$ by incubating fibroblasts in phosphate-free DMEM for 1 hr, followed by a 4 hr incubation with phosphate-free DMEM containing 0.2 mCi/mL $[^{32}\text{P}]$. RSK2 was activated during the last hour of labeling by addition of 0.1 ng/mL hEGF. 5-HT$_{2A}$ receptors were immunopurified from cell lysates in the presence of phosphatase inhibitors using M2 FLAG affinity resin as described previously (209). Receptor purification was verified via Western blot and equal amounts of receptor were separated on a 10% SDS-PAGE gel, dried, and imaged (Kodak BioMax MR, Eastman Kodak).
2.2.11 Fluorometric Imaging Plate Reader (FLIPR\textsuperscript{Tetra}) Analysis of Intracellular Ca\textsuperscript{2+} Release

Intracellular calcium release was measured using a FLIPR\textsuperscript{Tetra} and calcium assay kit (Molecular Devices, Sunnyvale, CA). Briefly, 30,000 cells were plated into black-wall, clear-bottom 96-well tissue culture plates (Greiner Bio-One, Monroe, NC) in dialyzed culture medium (DMEM, 5% FBS dialyzed to <0.05 nM 5-HT, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin). The cell culture medium was replaced with calcium assay buffer (20 mM HEPES, 1X HBSS, 2.5 mM probenecid, and calcium assay reagent, pH 7.4), incubated for 1 hr at 37°C, and equilibrated to room temperature before the assay. For growth factor desensitization experiments cells were incubated in serum-free medium (DMEM, 0.1% BSA, 100 units/mL penicillin, and 100 μg/mL streptomycin) for 24-40 hr before addition of calcium assay buffer. Concentrated stock solutions of growth factors were stored in single use aliquots at -20°C. For time course experiments, growth factors were diluted in serum-free medium (for 2-4 hr time points) or calcium assay buffer (for 30-60 min time points) and incubated for the indicated times. The FLIPRTetra was programmed to add agonist approximately 10 seconds after establishing baseline relative fluorescence unit (RFU) values (excitation 470-495, emission 515-575 nm). RFU values were collected every second for 5 min and the average baseline values were subtracted from maximum RFU values. Baseline-subtracted values were normalized to cell number, expressed relative to the maximal wild-type 5-HT response, and analyzed by nonlinear regression (Graphpad) as described below.
To normalize receptor expression we treated RSK2+/+ and RSK2/- cells with EEDQ (1-100 µM) for 30 min at 37°C prior to incubation with FLIPR assay buffer. Cells were also treated in parallel with EEDQ (1-100 µM) prior to preparation of membranes for quantification of receptor expression via radioligand binding. Concentrations of EEDQ that produced equal levels of receptor expression in both cell lines were compared for their agonist responses in the FLIPR assay.

2.2.12 Analysis of Inositol Phosphates

2.2.12.1 Analysis of Inositol-1,4,5-Trisphosphate

The [³H] Biotrak Assay System (Amersham TRK1000) was used exactly as described by the manufacturer to specifically detect perchloric acid-extracted inositol-1,4,5-trisphosphate (IP₃). Briefly, cells were plated into 6-well plates (1x10⁶ cells/well) using dialyzed culture medium (DMEM, 5% FBS dialyzed to <0.05 nM 5-HT, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 µg/mL streptomycin) and incubated 37°C. The next day the medium was removed and replaced with 0.45 mL serum-free OptiMEM containing 10 mM LiCl. At the appropriate time (i.e. 10 sec, 30 sec, 60 sec, 90 sec, 120 sec) 0.05 mL of 10X drug (100 µM 5-HT, diluted into OptiMEM) was added and the plates were incubated at 37°C. Accumulation of IP₃ was stopped by immediately placing the plates on ice and adding 125 µL of ice cold 20% perchloric acid. After 20 min on ice, each well was scraped into a low retention 1.5 mL tube and centrifuged 2000 x g for 15 min at 4°C. The supernatants were transferred to fresh low retention 1.5 mL tubes containing 10 µL of pH indicator (Fisher) and neutralized with 5 M KOH + 60 mM HEPES (~pH 7). The IP₃-containing supernatants were separated from the precipitated
KClO₄ via centrifugation and stored in fresh low retention 1.5 mL tubes at -80°C. IP₃ was analyzed exactly as described by the manufacturer wherein NaOH was used to release IP₃ from the IP₃ binding protein followed by neutralization with acetic acid. Radioactivity was quantified by liquid scintillation counting (3a70B cocktail, RPI) and the amount of IP₃ present was extrapolated from a standard curve.

2.2.12.2 Analysis of Inositol Phosphate

The accumulation of inositol phosphate (IP) in experiments probing the importance of RSK2 kinase activity in regulating 5-HT₂A receptor signaling (performed by Dr. Douglas J. Sheffler and presented here for completeness) was measured via anion-exchange chromatography as detailed elsewhere (191,193). All other measurements of IP in this thesis were made using the scintillation proximity assay (SPA) method of Bourdon et al. (301). Briefly, 30,000 cells were plated into 96-well tissue culture plates in dialyzed culture medium. The cells were briefly inositol-starved (1.5 hr) and incubated for 18 hr at 37°C with labeling medium (inositol-free BME, 5% dialyzed FBS, and 0.01 µCi/µL [³H]myo-inositol). The labeling medium was removed and agonists diluted in assay buffer (1X HBSS, 24 mM NaHCO₃, 11 mM glucose, and 35 mM LiCl, pH 7.4) were added to the cells for 1 hr at 37°C. The assay was terminated by adding 30 µL of 50 mM formic acid for 1 hr and the supernatant was incubated with 0.2 mg yttrium silicate beads (YSi, Amersham). Radioactivity was measured by scintillation counting (Wallac Microbeta TriLux, PerkinElmer). Baseline-subtracted values were normalized to cell number, expressed relative to the maximal wild-type 5-HT response, and analyzed by nonlinear regression (Graphpad) as described below.
2.2.13 Generation and Validation of High Content Immunofluorescence Assays

A novel high content microscopic technique was developed to produce concentration response curves for ERK1/2 phosphorylation in RSK2+/+ and RSK2-/MEFs. Specifically we developed an extremely versatile triple fluorophore labeling method that uses information gained from nuclear (320 nm) and plasma membrane (488 nm) staining to generate cellular masks (i.e. segmentation), which are then used to quantify the fluorescence intensity of the third fluorophore (signal channel, 594 nm) representing the protein of interest (i.e. phosphorylated ERK1/2). RSK2+/+ and RSK2-/MEFs were plated onto 0.2% gelatin coated black-wall, clear-bottom 384-well tissue culture plates (Greiner Bio-One, Monroe, NC) in dialyzed culture medium at a density of 25,000 and 15,000 cells/well, respectively. One day later the cells were washed with serum-free medium (DMEM, 100 units/mL penicillin, and 100 μg/mL streptomycin) and incubated in serum-free medium for 18 hr. For initial time course experiments (0-30 min) 5-HT2A receptors were stimulated with 10 μM drug. For all subsequent experiments I used the liquid handling capability of a FLIPR Tetra to simultaneously dispense 4X concentrated drug dilutions (final concentration range of 10 μM to 10 pM, performed in duplicate) into 384-well plates. 5-HT2A receptors were stimulated for 5 min at 37ºC and immediately placed on ice, rinsed with ice-cold PBS wash buffer (PBS + 0.5 mM CaCl2, pH 7.4), and incubated with fixative (4% paraformaldehyde, PBS + 0.5 mM CaCl2, pH 7.4) to terminate receptor signaling. After 30 min at 25ºC the cells were washed and treated with 0.3% Triton X-100 for 30 min on ice. The permeabilized cells were incubated with blocking buffer (5% normal goat serum, PBS +0.5 mM CaCl2, pH 7.4) for
1 hr at 25ºC and then incubated with blocking buffer containing a phospho-ERK1/2 specific antibody (Thr202/Tyr204, 1:1000; Cell Signaling Technology, Inc., Danvers, MA) for 18 hr at 4ºC. The following day the cells were extensively washed and incubated for 1 hr at 25ºC with blocking buffer containing Hoechst (5 μg/mL), Concanavalin A conjugated to Alexa Fluor488 (20 μg/mL), and a goat anti-rabbit secondary antibody conjugated to Alexa Fluor594 (1:200). The cells were extensively washed and incubated with fixative for 20min at 4ºC. Plates were then sealed and stored at 4ºC in wash buffer before imaging.

Imaging was performed on a BD Pathway 855 High Content Bioimager (BD Biosciences, San Jose, CA) using the Olympus UAPO40X/340 objective. We developed a workflow that employed laser autofocus, triple excitation/emission parameters (nuclear-380/435 nm, plasma membrane-488/515 nm, signal channel-555/645 nm), and 3x3 imaging to produce superimposed nuclear, plasma membrane, and signal channel images. Images were exported to Cell Profiler (Broad Institute Imaging Platform, Cambridge, MA) for segmentation and analysis. Specifically, we developed a pipeline within Cell Profiler that produces reliable cell segmentation wherein Hoechst and Concanavalin A intensities are used to generate nuclear, whole cell, and cytoplasmic cell masks (Figure 2-9). As a result we could measure the 594 nm intensity within defined cellular regions. Baseline-subtracted, average whole cell 594 nm intensity values corresponding to ERK1/2 phosphorylation were then analyzed by nonlinear regression (Graphpad Prism) and expressed relative to the E_{max} for 5-HT in RSK2+/+ MEFs (see below).
Figure 2-9. Development of a novel triple-label high content microscopic assay in 384-well plates (A) to rapidly measure ERK1/2 phosphorylation in RSK2+/+ and RSK2−/− MEFs (B). A, Diagram showing stepwise generation of nuclear (Hoechst-380 nm), whole cell (Concanavalin A-488 nm), and cytoplasmic cell masks subsequently used to measure fluorescence intensity of the signal channel (594 nm) in distinct cellular regions. This approach incorporates the nearly limitless application of state-specific antibodies (e.g. phospho-specific) and fluorescently labeled proteins to measure various receptor responses. Moreover, segmenting the
cell into distinct regions provides us with the opportunity to extract a variety of signaling phenotypes with customizable CellProfiler image analysis software. B, I used this technology to generate full concentration response curves for ERK1/2 phosphorylation in RSK2+/+ and RSK2−/− MEFs after agonist treatment. The workflow shown here represents the steps used to measure ERK1/2 phosphorylation (594nm, see representative images) within whole cell masks after 5-HT treatment. Nonlinear regression curve fits (bottom) show the quantified data (mean +/- SEM) for 5-HT-induced ERK1/2 phosphorylation in RSK2+/+ (■) and RSK2−/− (○) MEFs. Also shown are representative results (bar graph) wherein the 5-HT2A selective antagonist MDL100907 (1 µM) blocked the response to 5-HT (~EC80 concentration).

2.2.14 Analysis of Intracellular Ca2+ Release in Primary Cortical Neurons

Neurons were isolated and cultured as described above. Forty-eight hours after lentivirus infection, each well was imaged for total GFP fluorescence using the BD Pathway 855 high content imaging microscope equipped with environmental control. Ca2+ flux was imaged and determined using the FLIPR calcium assay kit (Molecular Probes, cat#R8033) as detailed by the manufacturer. In brief, prior to live cell imaging, cells were washed 1x with PBS followed by 1 hr incubation with calcium assay buffer (20 mM HEPES, 1X HBSS, 2.5 mM probenecid, 0.57 mM ascorbic acid, and calcium assay reagent, pH 7.4). Assays using growth factors were performed similarly except that growth factor was added to the dye loading step. Cells were maintained at 37°C during the entire period of observation and were imaged for 20 sec prior to drug addition to obtain baseline dye fluorescence. The liquid handling capability of the BD Pathway 855
was used to add 10x drug (100 µM DOI, 10 µM final) and then fluorescence images were obtained for 120 sec. To control for subtle differences in receptor expression, Ca^{2+} responses were processed and normalized to GFP intensity/well using custom written macros for Excel (Microsoft, Redmond, WA) and Image J (U. S. National Institutes of Health, Bethesda, Maryland).

2.2.15 Mouse Behavioral Experiments

2.2.15.1 Locomotion

RSK2-/− mice (C57BL/6J) were generated and kindly supplied by Dr. André Hanauer (Institute of Genetics, and Molecular and Cellular Biology, Illkirch, CEDEX, France). All experiments were approved by the Institutional Animal Care and Use Committee at the University of North Carolina, Chapel Hill. Mice were housed under standard conditions –12 hr light/dark cycle and food and water ad libitum. For all behavioral experiments, 12-week old mice were acclimated to the testing facility 20 min before testing. Exploratory activity in a novel environment was assessed by 1 hr trials, given on separate days, in an open field chamber (40 cm x 40 cm x 30 cm) crossed by a grid of photobeams (VersaMax system, AccuScan Instruments). Counts were taken of the number of photobeams broken during the trial in 5 min intervals, with separate measures for horizontal activity and time spent in the center region of the activity chamber.

2.2.15.2 Head Twitch Response (HTR)

The head twitch response elicited after administering hallucinogens to mice consists of a characteristic, rapid, rotational flick of the head, ears, and neck that is easily
scored and is distinguishable from other head movements such as head shakes or head jerks. Mice were injected intraperitoneal (i.p) with one of three doses of DOI: 0.3, 0.75, or 1 mg/kg. Considerable freezing behavior (i.e. immobility) was observed in doses above 1.0 mg/kg DOI and these animals were not included in the analysis. The number of head twitches was counted and recorded in 5 min bins for the 1 hr period immediately following injection. A subset of the injections (N=6) were counted by two blinded observers. A comparison of the results produced by the two different observers was not significantly different, therefore all remaining head twitch experiments were performed by one blinded observer.

2.2.16 Statistical Analysis of In Vitro Kinase Assays, Second Messenger Assays, High Content ERK1/2 Phosphorylation Assays, and Mouse Behavioral Experiments

Statistical significance (defined as $p<0.05$) of the in vitro kinase data (both phosphoimager and Western blot band quantification) was determined by the one-tailed paired t test. In signaling experiments, the F test was used to determine the statistical significance (defined as $p<0.05$) of the fit parameters potency ($EC_{50}$) and efficacy ($E_{max}$) for $Ca^{2+}$ release, IP accumulation, and ERK1/2 phosphorylation. Statistical significance (defined as $p<0.05$) of IP$_3$ and ERK1/2 time course data was determined by the one-tailed paired t test. The two-tailed paired t test was used to determine the statistical significance (defined as $p<0.05$) of FLIPR $Ca^{2+}$ traces in the growth factor desensitization experiments. The mouse locomotion experiments were analyzed via repeated measures ANOVA (significance defined as $p<0.001$) by Sheryl Moy (Mouse
Behavioral Phenotyping Laboratory, UNC, Chapel Hill). Head twitches were analyzed via the two-tailed paired t test with significance set as $p<0.05$.

2.2.17 Generation of Statistically Rigorous Rank Orders of Efficacy

Relative agonist efficacies were analyzed using one-way analysis of variance (ANOVA, significance set as $p<0.05$) and significant differences between groups were subsequently identified via the Tukey-Kramer unplanned multiple comparisons test (significance set as $p<0.05$) (Graphpad). Mean relative agonist efficacies were then arranged in descending order and assigned to statistically homogeneous groups such that significant differences in rank order (set as $p<0.05$) were denoted by changes in group membership.
3.1 Introduction and Rationale

As discussed in Chapter 1, the 5-HT$_2A$ receptor belongs to the GPCR superfamily which encompasses molecular targets for an extreme diversity of endogenous and exogenous ligands that are essential for nearly every physiological process. Pioneering studies analyzing β$_2$AR signaling, trafficking, and protein interactions following agonist stimulation have resulted in a ‘classical’ model of GPCR regulation wherein agonists initiate a cascade of events to attenuate signaling including rapid receptor phosphorylation by GRKs, recruitment of arrestins, sequestration in clathrin-coated pits, and internalization (103). Indeed, extensive studies focusing on the GRK-arrestin pathway and the second messenger-dependent protein kinase pathways (i.e. PKA and PKC) suggest that direct GPCR phosphorylation remains the predominant mechanism for rapidly attenuating the signaling of many GPCRs (179,180).

Several studies have demonstrated that PKC modulates 5-HT$_2A$ receptor signaling in vivo and in vitro. Our early studies (182) showed that activation of PKC by phorbol dibutyrate inhibited 5-HT$_2A$-mediated signaling in rat aorta. Many subsequent studies in a variety of cellular contexts including rat brain, NIH 3T3 fibroblasts, and CHO cells have replicated these observations (183-188). In addition to PKC, recent reports suggest that calmodulin-dependent protein kinase II and GRK2/3 regulate 5-HT$_2A$ signaling (188,192), although the role of GRKs seems to be cell-specific (193). These studies clearly demonstrated that selected kinases modulate 5-HT$_2A$ receptor function; however,
in the absence of direct evidence showing receptor phosphorylation the site(s) of action and their mechanisms remain unknown. As hinted at here, additional kinases have also been shown to phosphorylate GPCRs and it is likely that many yet to be discovered kinases regulate GPCR signaling (181).

Recently our laboratory discovered that RSK2, a downstream effector of the ERK/MAPK pathway, regulates the signaling of several GPCRs including 5-HT$_{2A}$, P2Y-purinergic, PAR-1 thrombinergic, β$_1$AR, and bradykinin-B receptors (172). As discussed in Chapter 1, RSK2 is a well-characterized member of the RSK family of multifunctional ERK effectors (RSKs 1-4) and has been shown to phosphorylate a wide variety of cytoplasmic and nuclear proteins. We recently showed that RSK2 interacts with the 5-HT$_{2A}$ i3 loop within a conserved region containing an RSK2-like consensus phosphorylation motif (\textsuperscript{275}R-A-K-L-A-S\textsuperscript{280}) (243). Importantly, RSK2 modulated 5-HT$_{2A}$ receptor signaling independent of changes in 5-HT$_{2A}$ receptor sub-cellular distribution, global G protein function, and without altering the expression of any genes known to be involved in serotonergic signal transduction. These findings implied that RSK2 acts proximal to receptor activation, at the level of receptor-G protein coupling, perhaps via direct phosphorylation of 5-HT$_{2A}$ receptors. To test the hypothesis that RSK2 regulates 5-HT$_{2A}$ receptor signaling via direct phosphorylation, I devised a multidisciplinary approach that incorporated \textit{in vitro} kinase assays, tandem mass spectrometry (MS), site-directed mutagenesis, phospho-specific Western blot analysis, metabolic labeling studies, and whole-cell signaling experiments.
3.2 RSK2 Regulates both Rapid and Prolonged 5-HT$_{2A}$ Receptor Signaling

It is known that agonist activation of the 5-HT$_{2A}$ receptor leads to phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to produce the labile inositol phosphates inositol-1,4,5-trisphosphate (IP$_3$) and inositol-1,4-bisphosphate (IP$_2$), followed by accumulation of the terminal degradation product inositol-1-phosphate (IP) in the presence of LiCl to block inositol-1-phosphatase (156,158,182,302). Our laboratory has previously shown that RSK2 regulates 5-HT$_{2A}$-mediated IP accumulation during a 1hr incubation with 5-HT (172). However, the ability of RSK2 to regulate 5-HT$_{2A}$ receptor-mediated IP$_3$ accumulation during short agonist treatments has not been determined. In this set of experiments I wanted to address this by directly measuring the rapid production of IP$_3$ in RSK2+/+ and RSK2-/- MEFs stably expressing rat 5-HT$_{2A}$ receptors following 10, 30, 60, and 90 sec stimulation with 10 µM 5-HT. As shown in Figure 3-1A, maximal accumulation of IP$_3$ occurred within 10 sec, consistent with early reports detecting 5-fold increases in IP$_3$ as early as 5 sec after 5-HT treatment (303). Similar to what I observed with prolonged IP accumulation occurring over 1hr (Figure 3-1B), genetic deletion of RSK2 significantly potentiated 5-HT-induced IP$_3$ production at all time points. These data clearly show that RSK2 regulates 5-HT$_{2A}$ signaling during extremely short periods of activation and is consistent with our previous data showing significant potentiation of rapid Ca$^{2+}$ responses (172). Moreover, these early time points are most likely not confounded by compensatory regulatory mechanisms (e.g. GRK-mediated phosphorylation and arrestin binding), thus suggesting that 5-HT$_{2A}$ receptors are pre-desensitized (i.e. ‘tonic brake’) in the presence of RSK2, perhaps via direct phosphorylation.
Figure 3-1. RSK2 regulates both rapid and prolonged 5-HT\textsubscript{2A} receptor signaling in MEFs. Time course experiments measured inositol-1,4,5-trisphosphate (IP\textsubscript{3}) (A) and inositol phosphate (IP) (B) accumulation in RSK2+/+ and RSK2-/- MEFs expressing FLAG 5-HT\textsubscript{2A} receptors following 10 µM 5-HT treatment. A, Rapid (0-90 sec) 5-HT-mediated IP\textsubscript{3} production as measured by a radioreceptor assay was significantly potentiated in RSK2-/- MEFs (○) when compared to RSK2+/+ MEFs (■). Shown here are results from three independent experiments (mean +/- SEM); *p<0.05. B, Prolonged (0-60 min) 5-HT treatment led to increased accumulation of [\textsuperscript{3}H]-IP in RSK2-/- MEFs (○) when compared to RSK2+/+ MEFs (■). Shown here are results from three independent experiments (mean +/- SEM).
3.3 RSK2 Kinase Activity is Essential for Negatively Regulating 5-HT$_{2A}$ Receptor Signaling

Control studies have indicated that this negative regulation occurs independently of alterations distal to receptor activation such as changes in $G_{aq}$ activity and receptor trafficking (172). Therefore we focused our initial efforts on determining whether RSK2 regulates 5-HT$_{2A}$ signaling proximal to receptor activation, perhaps by directly phosphorylating the 5-HT$_{2A}$ receptor. Please note that these studies were carried out by Dr. Douglas J. Shefler and are presented here since 1) they were published with the work in Chapter 3 (290) and 2) they are critical to the synthesis of this thesis.

Since the intracellular loops and carboxy-terminus of the 5-HT$_{2A}$ receptor contain an estimated 37 Ser or Thr phospho-acceptor sites (191), the most logical approach was to initially determine if RSK2 kinase activity was necessary for regulating 5-HT$_{2A}$ signaling. In the ‘add-back’ approach outlined here, N-terminal (K100A) and C-terminal (K451A) kinase-deficient RSK2 mutants were stably re-introduced, alongside wild-type RSK2, into RSK2/-/- MEFs and judged by their ability to normalize signaling compared to RSK2+/+ MEFs (Figure 3-2A). Importantly, mutation of the critical Lys in the ATP binding site of each kinase domain was previously reported to inhibit catalytic activity (304). As shown in Figure 3-2B, re-introduction of wild-type RSK2 into RSK2/-/- MEFs normalized signaling to levels indistinguishable from RSK2+/+ MEFs. However, the K451A C-terminal kinase-deficient mutant, despite expression similar to endogenous RSK2 levels, failed to normalize signaling (Table 3-1). These data, alongside evidence showing that the kinase-deficient mutants retain interactions with 5-HT$_{2A}$ receptors (Figure 3-2C), suggest that RSK2 kinase activity is essential for regulating 5-HT$_{2A}$
receptors. Moreover, these results demonstrate that a direct physical interaction between 5-HT$_{2A}$ receptors and RSK2 is insufficient to modulate signaling, since kinase-dead mutants interact with the 5-HT$_{2A}$ receptor but do not rescue the RSK2/- signaling phenotype.

Table 3-1

Effects of ectopically expressed wild-type and kinase-dead RSK2 on endogenous 5-HT$_{2A}$ receptor signaling in mouse embryonic fibroblasts

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Agonist Potency $^a$ (EC$<em>{50}$ (pEC$</em>{50}$ ± SEM $^b$))</th>
<th>Relative Agonist Efficacy $^a$ % E$_{max}$ +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSK2 +/- fibroblasts</td>
<td>411 nM (6.39 ± 0.12)</td>
<td>106 ± 5</td>
</tr>
<tr>
<td>RSK2 -/- fibroblasts</td>
<td>515 nM (6.29 ± 0.28)</td>
<td>181 ± 2 $^c$</td>
</tr>
<tr>
<td>RSK2 -/- wild-type RSK2 stable</td>
<td>368 nM (6.44 ± 0.21)</td>
<td>107 ± 9</td>
</tr>
<tr>
<td>RSK2 -/- RSK2-K451A stable</td>
<td>436 nM (6.36 ± 0.13)</td>
<td>158 ± 8 $^c$</td>
</tr>
</tbody>
</table>

$^a$Agonist potencies (EC$_{50}$) and efficacies (E$_{max}$) were determined for agonist-mediated inositol phosphate (IP) accumulation. The results represent the average of four independent experiments.

$^b$pEC$_{50}$ values are represented as –log of EC$_{50}$ in M.

$^c$Statistically different from RSK2 +/- fibroblasts, $p<0.0$

* This work was performed by Douglas J. Sheffler
Figure 3-2. RSK2 kinase activity is essential for negatively regulating 5-HT$_{2A}$ receptor signaling. In order to determine if RSK2 kinase activity is required for regulation of 5-HT$_{2A}$ receptor signaling, an ‘add-back’ approach was developed by Dr. Douglas J. Sheffler wherein wild-type or kinase-deficient (K100A or K451A) RSK2 constructs (A) were expressed in RSK2-/- MEFs for rescue experiments (B) or HEK293T cells for co-immunoprecipitations (C). A, Western blot analysis of cell
lysates using an antibody specific to RSK2 shows similar RSK2 expression levels in RSK2-/- wild-type and RSK2-/- K451A fibroblast cell lines. B, Stimulation of fibroblast cell lines with 5-HT (1 nM-100 µM) shows concentration-dependent accumulation of [3H]-phosphoinositides from [3H]-PIP2 hydrolysis. Disruption of kinase activity in RSK2-K451A fibroblasts (▼) failed to significantly attenuate maximal 5-HT2A signaling to levels seen with fibroblasts expressing endogenous RSK2 (▲) or RSK2 -/- fibroblasts ectopically expressing wild-type RSK2 (●). The RSK2-K451A fibroblasts (▼) signaled similarly to RSK2-/- fibroblasts (■), thus suggesting that RSK2 kinase activity is essential for 5-HT2A regulation. These data were normalized to dpm/mg total protein where RSK2 +/- fibroblast maximal signaling is set to 100% and represent four independent experiments (mean +/- SEM); *p<0.05 when compared to RSK2 +/- fibroblasts. C, FLAG-5-HT2A receptors were co-transfected with wild-type or kinase-dead RSK2 constructs fused to GFP in HEK293T cells. GFP was used in this case to separate endogenous RSK2 from exogenous RSK2 (arrow, lower panel). Both wild-type and kinase-deficient RSK2 proteins co-immunopurified with FLAG 5-HT2A as detected by an antibody against GFP (arrow, top panel). Shown are representative immunoblots from a single experiment that was replicated three times with equivalent results.

3.4 RSK2 Phosphorylates the 5-HT2A Receptor In Vitro

Given that RSK2 interacts with 5-HT2A receptors in vitro and the 5-HT2A i3 loop contains an RSK2-like consensus site, the most likely explanation for these results is that RSK2 modulates 5-HT2A signaling via receptor phosphorylation. I initially tested this
hypothesis via *in vitro* kinase assays incorporating purified, activated RSK2 and purified 5-HT$_{2A}$ receptors. Potentially a large number of phosphorylation events are possible given the number of Ser/Thr phopho-acceptor sites in the cytoplasmic regions of the 5-HT$_{2A}$ receptor, especially the i3 loop (Figure 3-3A). As shown in Figure 3-3B, RSK2 autophosphorylation demonstrated that the *in vitro* assay conditions retained RSK2 kinase activity (arrow ~88 kDa), as reported by others (305). Incubation of purified 5-HT$_{2A}$ receptors with activated RSK2 and [γ-$^{32}$P]-ATP resulted in robust [$^{32}$P]-phosphate incorporation into the 5-HT$_{2A}$ receptor (arrow at ~62 kDa) relative to control assays lacking RSK2 (Figures 3-3B and C). Importantly, the selective RSK inhibitor SL0101, used at a concentration which inhibits >75% of RSK2 N-terminal kinase activity (276), blocked phosphorylation of the receptor (Figure 3-3C). Thus incubation with 10 μM SL0101 attenuated 5-HT$_{2A}$ phosphorylation to levels approaching the control assay lacking RSK2 (175 +/- 41% vs. 474 +/- 148% increase in [$^{32}$P]-phosphate incorporation in the presence and absence of SL0101, respectively; *p*<0.05, N=4). We further confirmed that inhibition of the N-terminal kinase domain of RSK2 attenuated 5-HT$_{2A}$ receptor phosphorylation by obtaining identical results with 100 nM of the selective RSK inhibitor BI-D1870 (data not shown), a concentration which inhibits 98% of RSK2 N-terminal kinase activity (299). Since the N-terminal kinase domain of RSK2 is responsible for substrate phosphorylation, a significant decrease in 5-HT$_{2A}$ phosphorylation upon application of these specific RSK inhibitors supports our conclusion that activated, purified RSK2 phosphorylates the 5-HT$_{2A}$ receptor *in vitro*. 
Figure 3-3. RSK2 phosphorylates the 5-HT\textsubscript{2A} receptor \textit{in vitro}. For these experiments purified FLAG-His\textsubscript{6} 5-HT\textsubscript{2A} receptors (A) were phosphorylated \textit{in vitro} using purified, activated RSK2 (B-C). A, Diagram illustrating design of the tandem affinity-tagged 5-HT\textsubscript{2A} receptor and alignment of rat, human, and mouse i3 loop amino acid sequences. Potential Ser or Thr phosphorylation sites are underlined and the RSK2-like consensus phosphorylation motif is boxed with the optimal sequence shown above. B, The FLAG-His\textsubscript{6} 5-HT\textsubscript{2A} receptor was \textit{in vitro} phosphorylated as described in MATERIALS AND METHODS. The top panel is a representative phosphorimage showing RSK2-mediated incorporation of [\textsuperscript{32}P]-phosphate into the 5-HT\textsubscript{2A} receptor (arrow at \~62 kDa). Presence of the specific
RSK inhibitor 10 μM SL0101 blocked [32P]-phosphate incorporation into the 5-HT$_{2A}$ receptor. Western blot analysis of the samples loaded for phosphorimager detection (lower panels) confirmed the components of each in vitro kinase assay. C, Quantification of 5-HT$_{2A}$ receptor phosphorylation shown in (B). The sum pixel intensity values from phosphorimager bands were normalized to control assays lacking RSK2 (set to 100%). These data show that SL0101 significantly decreased RSK2-mediated [32P]-phosphate incorporation into the 5-HT$_{2A}$ receptor. Shown here are results from four independent experiments (mean +/- SEM), *p<0.05.

3.5 RSK2 Phosphorylates the 5-HT$_{2A}$ Receptor I3 Loop In Vitro

It is known that the 5-HT$_{2A}$ i3 loop interacts with RSK2 within a region containing a conserved RSK2-like consensus phosphorylation motif ($^{275}$R-A-K-L-A-$^{280}$, boxed in Figure 3-3A). Thus I next determined if RSK2 can phosphorylate the i3 loop using in vitro kinase assays incorporating [$\gamma$-32P]-ATP, purified i3 loop peptide (amino acids 252-328, Figure 3-3A), and purified, activated RSK2. Consistent with results using the full-length 5-HT$_{2A}$ receptor, activated RSK2 robustly incorporated [32P]phosphate into the purified i3 loop peptide relative to control assays lacking RSK2 (Figure 3-4A). Phosphorimager quantification in Figure 3-4B shows that 10 μM SL0101 attenuated phosphorylation (440 +/- 109% vs. 1360 +/- 186% increase in [32P]-phosphate incorporation in the presence and absence of SL0101, respectively; p<0.05, N=3). These data show that the i3 loop is an RSK2 substrate in vitro. I further determined the kinetics of i3 loop phosphorylation by measuring [32P]-phosphate incorporation into i3 loop peptide (2.3 μM to 140 μM). These experiments were performed under initial velocity
conditions in which the i3 loop peptide substrate was not depleted and the detection system was not saturated (data not shown, see Chapter 2 MATERIALS AND METHODS). As shown in Figure 3-4C, RSK2 phosphorylated the i3 loop peptide with saturable kinetics resulting in a maximal reaction rate ($V_{\text{max}}$) equal to 0.099 +/- 0.012 µmolP·min$^{-1}$·mg$^{-1}$ RSK2 and an estimated $K_m$ of 81.8 +/- 19.6 µM. These results demonstrate that RSK2 robustly phosphorylates the 5-HT$_2A$ receptor i3 loop in vitro with an affinity similar to that previously reported for the RSK2 peptide substrate K-K-R-N-K-T-L-S-V-A (243).
Figure 3-4. RSK2 phosphorylates the 5-HT$_2$A receptor i3 loop in vitro. For these experiments purified i3 loop peptides were phosphorylated in vitro with purified, activated RSK2. A, The i3 loop peptide was in vitro phosphorylated as described in
Chapter 2 MATERIALS AND METHODS. The top panel is a representative phosphorimage showing RSK2-mediated incorporation of $[^{32}P]$-phosphate into the i3 loop peptide (arrow at ~6 kDa). The arrow at ~83 kDa denotes RSK2 autophosphorylation. The lower panels show Western blot validation of RSK2 and i3 loop peptide mobilities. B, Quantification of 5-HT$_{2A}$ receptor i3 loop phosphorylation shown in A. The sum pixel intensity values from phosphorimager bands were normalized to control assays lacking RSK2 (set to 100%). These data show that SL0101 significantly decreased RSK2-mediated $[^{32}P]$-phosphate incorporation into the i3 loop peptide. Shown here are results from three independent experiments (mean +/- SEM); *$p<0.05$. C, Kinetic analysis of i3 loop phosphorylation. In vitro kinase assays using increasing concentrations of i3 loop peptide (2.3 μM to 140 μM, inset) were performed under initial velocity conditions. Radioactive bands were excised and quantified by liquid scintillation counting. Background subtracted values were plotted against i3 loop peptide concentrations and fit to a one-site hyperbolic equation to determine $V_{max}$ and $K_m$.

3.6 RSK2 Specifically Phosphorylates the Conserved Residue Ser314 within the 5-HT$_{2A}$ Receptor I3 Loop In Vitro

In order to validate the in vitro data and to otherwise rule out stochastic, non-specific phosphorylation events which can occur with in vitro kinase assays, I searched for phospho-acceptor sites within the in vitro phosphorylated 5-HT$_{2A}$ receptor i3 loop. The 5-HT$_{2A}$ receptor i3 loop contains 18 potential phosphorylation sites for Ser/Thr kinases (underlined in Figure 3-3A), and, conceivably, any of these sites could be phosphorylated. Therefore I chose the unbiased approach of tandem MS to efficiently
explore i3 loop phosphorylation space. The sequence coverage for trypsin digestion of the i3 loop peptide was 81% (underlined in Figure 3-5A) and we were unable to account for only one Ser and one Thr located in the i3 loop N-terminus. Analysis of the CID spectra obtained from the kinase-treated i3 loop peptide found a total of four peptides that corresponded to putative phosphorylation sites. The first site, corresponding to Ser314 (S314, 100% conserved among rat, human, and mouse 5-HT$_2$A receptors, Figure 3-3A), was identified in two consecutive experiments from the peptides $^{311}$TMoQpSISNEQK$^{320}$ and $^{310}$RTMoQpSISNEQK$^{320}$ (where Mo is an oxidized Met and pS represents phosphoSer). The CID spectrum of the $^{311}$TMoQpSISNEQK$^{320}$ peptide is shown in Figure 3-5A. This spectrum has the expected abundant ion from the loss of H$_3$PO$_4$ (-98 Da) and, particularly in the case of the $^{311}$TMoQpSISNEQK$^{320}$ peptide, sufficient information to establish the site of phosphorylation. Mass chromatograms were plotted for these ions that clearly showed the detection of both forms of the phosphopeptide in the kinase-treated sample and the absence of the peptides in the control sample without kinase (data not shown). A second minor phosphorylation site (Ser280, S280) was also observed that corresponds to the phosphopeptide $^{278}$LApSFSFLPQSSLSEK$^{293}$ which contains the phosphorylated Ser in the RSK2-like consensus phosphorylation motif (data not shown). Again, a prominent ion from the loss of H$_3$PO$_4$ was observed. Furthermore, significant sequence-specific ions were observed to verify the correct peptide. The exact site of phosphorylation is less clear and was determined with an MS$^3$ experiment in which the fragment ion from the loss of H$_3$PO$_4$ was refragmented and a pair of low abundance y-ions was observed that corresponded to the expected dehydro-Ala. However, the significance of this site is questionable because this phosphopeptide was
only detected in one of the experiments and was present in both the kinase-treated sample and control sample without kinase indicating that its formation was independent of kinase treatment. Two exceedingly low abundance phosphopeptides were also identified, 264EATLCVSDLSTR275 and 298SIHREPGSYAGR309 (asterisks, Figure 3-5A). These spectra contained only a limited amount of sequence information due to the abundant ion from the loss of H3PO4. These peptides were not seen in all of our experiments and this lack of reproducibility would lessen the potential significance of these sites. These sites are reported here for completeness.

The MS phosphorylation assignment was verified using two independent methods. First I used site-directed mutagenesis to generate Ser to Ala mutants at both MS assigned sites (S280A and S314A), thus rendering each site phosphorylation-deficient. As shown in Figure 3-5B and quantified in Figure 3-5C, activated RSK2 incorporated [32P]phosphate into the wild-type and the S280A mutant peptides to a similar extent relative to control assays lacking RSK2 (978 +/- 213% and 1010 +/- 152% increase in [32P]-phosphate incorporation for wild-type and S280A peptides, respectively; p>0.05, N=3). However, the S314A mutation completely abolished RSK2-mediated phosphorylation when compared to [32P]-phosphate incorporation for the wild-type peptide (95.0 +/- 10.9% vs. 978 +/- 213% increase in [32P]-phosphate incorporation for S314A and wild-type peptides, respectively; p<0.05, N=3). Taken together, results using phosphorylation-deficient i3 peptides support the robust MS assignment at Ser314 and show that the minor Ser280 site does not significantly contribute to phosphorylation in vitro. I also tested the phosphorylation sites found in the low abundance phosphopeptide
SIHREPGSYAGR<sup>309</sup> (S298A and S305A) and found that neither mutation inhibited RSK2 phosphorylation (Figure 3-5D).

Figure 3-5. RSK2 specifically phosphorylates Ser314 within the 5-HT<sub>2A</sub> receptor i3 loop. For these experiments purified 5-HT<sub>2A</sub> i3 loop peptides were phosphorylated <i>in vitro</i> with purified, activated RSK2 and phosphate incorporation was subsequently analyzed by MS (A) and <sup>32</sup>P-phosphate incorporation (B-C). A, Tandem MS analysis of <i>in vitro</i> phosphorylated wild-type i3 peptide identified Ser280 (S280) and Ser314 (S314) as putative phosphorylation sites (arrows, top
Several low abundance phosphopeptides were identified including \textsuperscript{264}EATLCVSDLSTR \textsuperscript{275} and \textsuperscript{298}SIHREPGSYAGR \textsuperscript{309} (potential phosphorylation sites denoted by asterisk and arrows). The CID spectrum of the reproducible and robust phosphopeptide \textsuperscript{311}TMoQpSISNEQK \textsuperscript{320} is shown where Mo represents oxidized Met and pS represents phospho-Ser\textsuperscript{314}. Although the CID spectrum contains an abundant ion due to the loss of H\textsubscript{3}PO\textsubscript{4} (-98 Da) that is characteristic of Ser phosphorylation, sufficient information to place the site of phosphorylation is also observed. Specifically the mass difference of 167 Da between y-series ions \textit{y}_6 and \textit{y}_7 corresponds to phospho-Ser\textsuperscript{314}. 

B, Site-directed mutagenesis of Ser\textsuperscript{280} (S\textsuperscript{280}A) and Ser\textsuperscript{314} (S\textsuperscript{314}A) indicates that RSK2 predominantly phosphorylates Ser\textsuperscript{314}. Western blot analysis of the samples loaded for phosphorimager detection (lower panels) confirmed the components of each \textit{in vitro} kinase assay. C, Quantification of i3 loop peptide phosphorylation shown in (B). The sum pixel intensity values from phosphorimager bands were normalized to control assays lacking RSK2 (set to 100\%). The S\textsuperscript{314}A mutation completely abolished [\textsuperscript{32}P]-phosphate incorporation into the i3 loop when compared to the wild-type peptide. Shown here are results from three independent experiments (mean +/- SEM); *p<0.05. D, Site-directed mutagenesis of two Ser residues located in the low abundance phosphopeptide \textsuperscript{298}SIHREPGSYAGR \textsuperscript{309} (Ser\textsuperscript{298}, S\textsuperscript{298}A and Ser\textsuperscript{305}, S\textsuperscript{305}A) indicates that these sites are not important for phosphorylation \textit{in vitro}. 

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3.7 The Phospho-Specific Antibody Against Ser314 Verifies Phosphorylation within the 5-HT$_{2A}$ Receptor I3 Loop.

I further verified the MS assignment by generating a phospho-specific antibody against phospho-Ser314 (Ser(P)-314) to probe in vitro kinase assays. As shown in Chapter 2 MATERIALS AND METHODS, the Ser(P)-314 Ab selectively detects ~10 ng of the phosphorylated antigenic peptide (bottom panel, Figure 3-6A). As shown in Figure 3-6A and quantified in Figure 3-6B, I detected a large increase in Ser(P)-314 Ab immunolabeling when the wild-type i3 loop peptide was incubated with activated RSK2. In support of the selectivity of the Ser(P)-314 Ab, and consistent with the Ala mutagenesis data, the phospho-Ser314-specific Ab failed to immunolabel the phosphorylation-deficient i3 loop peptide (i3-S314A) when compared with the wild-type i3 loop peptide (86.0 +/- 2.7% vs. 370 +/- 93% increase in control pixel intensity for the S314A and wild-type i3 peptides, respectively; $p<0.05$, N=3). Taken together, the MS, site-directed mutagenesis, and phospho-specific immunoblotting results unambiguously show that RSK2 specifically phosphorylates Ser314 within the 5-HT$_{2A}$ receptor i3 loop in vitro.
Figure 3-6. Phospho-specific antibody validation of Ser314 phosphorylation within the 5-HT$_2A$ receptor i3 loop. For these experiments purified wild-type and phosphorylation-deficient (i3-S314A) i3 loop peptides were phosphorylated in vitro with purified, activated RSK2 and subsequently immunoblotted with the phospho-S314-specific antibody (Ser(P)-314 Ab, bottom panel shows specificity). A, Ser(P)-314 Ab immunolabeling shows that activated RSK2 phosphorylates the wild-type i3 loop peptide (top panel, arrow at ~6 kDa). Inability of the Ser(P)-314 Ab to detect in vitro phosphorylated i3-S314A peptide indicates the lack of phosphorylation at this site and specificity of the Ab. Western blot analysis of the samples loaded for Ser(P)-314 Ab detection (lower panels) confirmed the components of each in vitro kinase assay. B, Quantification of Ser(P)-314 Ab immunolabeling shown in A. The sum pixel intensity values from i3 loop peptide bands (top, panel A) were normalized to
control assays lacking RSK2 (set to 100%). The S314A mutation completely abolished immunolabeling of the Ser(P)-314 Ab when compared to the wild-type peptide. Shown here are results from three independent experiments (mean +/-SEM); *p<0.05.

3.8 RSK2 Specifically Phosphorylates the Full-Length 5-HT$_2A$ Receptor at Ser314

The i3 loop peptide studies clearly demonstrate that activated RSK2 phosphorylates Ser314 in vitro. However, because this result was achieved using only the i3 loop fragment of the 5-HT$_2A$ receptor, I needed to validate this with intact, full-length receptor. Accordingly, I developed an unbiased strategy to probe RSK2 phosphorylation space similar to our i3 loop peptide experiments. Here I interrogated in vitro receptor phosphorylation using tandem MS, the Ser(P)-314 Ab, and [${}^{32}$P] metabolic labeling. As shown in the tandem MS spectrum of a doubly charged phospho-Ser-containing peptide $^{311}$TMQpSISNEQ$^{320}$ with $m/z$ of 623.5, phosphorylation at Ser314 was detected in the intact receptor (Figure 3-7A). Phosphorylation at Ser314 was confirmed by the detection of dehydro-Ala in the sequence due to the loss of H$_3$PO$_4$ (-98 Da) by β-elimination. Specifically, for the b-type series, b$_{4,9}$ fragments clearly show both the mass shifts of +80 Da and loss of H$_3$PO$_4$ (-98 Da); whereas b$_2$ and b$_3$ ions are unchanged upon comparison to the spectrum of the unmodified peptide. The y-type series ions y$_{2,6}$ show no modification; whereas for y$_{7,8}$ fragment ions both the mass shifts of +80 Da and loss of H$_3$PO$_4$ (-98 Da) are observed. Moreover, a mass difference of 167 Da between y-type series ions y$_7$ and y$_6$ and b-type series ions b$_3$ and b$_4$ was observed corresponding to a phospho-Ser. Therefore I can confidently assign phosphorylation to the conserved Ser314
in the intact 5-HT$_{2A}$ receptor (Figure 3-7B). An additional phosphorylation site (Ser305) was identified within the phosphopeptide $^{296}$SIHREPGSYAGR$^{309}$ but was not investigated since the S305A mutation in the i3 loop peptide failed to decrease phosphorylation (Figure 3-5D).
**Figure 3-7.** RSK2 phosphorylates the full-length 5-HT\textsubscript{2A} Receptor at S314 *in vitro.*

For these experiments, purified FLAG-His\textsubscript{6} 5-HT\textsubscript{2A} receptors were phosphorylated *in vitro* using purified, activated RSK2 and analyzed by tandem MS (A) to identify phosphorylation site(s) in the full-length receptor (B). A, Phosphopeptides derived from *in vitro* phosphorylated receptors were concentrated and analyzed as described in MATERIALS AND METHODS. Shown here is the tandem MS chromatogram of the phosphopeptide \textsuperscript{311}TMQpSISNEQK\textsuperscript{320}, where pS represents phospho-Ser314. Phosphorylation at Ser314 was confirmed by the detection of dehydro-Ala in the sequence due to the loss of the H\textsubscript{3}PO\textsubscript{4} (-98 Da) by β-elimination in both b- and y-series fragment ions. We also observed a mass difference of 167 Da between y-type series ions y\textsubscript{6} and y\textsubscript{7}, and b-type series ions b\textsubscript{3} and b\textsubscript{4} (double arrows, corresponds to phospho-Ser314. B, Diagram illustrating location of Ser314 (designated 6.26 according to (37), arrow) within the i3 loop in relation to Glu318 (6.30, arrow) which is necessary for maintaining the 5-HT\textsubscript{2A} receptor in an inactive conformation.

### 3.9 Validation of Ser314 Phosphorylation in 5-HT\textsubscript{2A} Receptors *In Vitro* and in MEFs.

I subsequently validated the MS assignment using 1) the Ser(P)-314 Ab to probe for phospho-Ser-314 under conditions that resulted in 5-HT\textsubscript{2A} receptor phosphorylation *in vitro*, and 2) \textsuperscript{32}P\textsubscript{i} metabolic labeling in intact fibroblasts stably expressing wild-type or phosphorylation-deficient (5-HT\textsubscript{2A}-S314A) 5-HT\textsubscript{2A} receptors. As shown in Figure 3-8A and quantified in Figure 3-8B, I detected a large increase in Ser(P)-314 Ab immunolabeling following *in vitro* phosphorylation of the 5-HT\textsubscript{2A} receptor with activated
RSK2. Additionally, I was able to attenuate Ser(P)-314 Ab recognition, and by extension 5-HT\(_{2A}\) phosphorylation, using the selective RSK inhibitor SL0101 (112 +/- 5% vs. 138 +/- 8% increase in control pixel intensity in the presence and absence of SL0101, respectively; \(p<0.05\), N=5). This result was internally consistent with our previous experiments using both the full-length receptor (Figure 3-3) and i3 loop peptide (Figure 3-4) in which the selective RSK inhibitor significantly attenuated \([^{32}P]\)phosphate incorporation.

Metabolic labeling of proteins has been the “gold standard” method for elucidating phosphorylation of numerous GPCRs in intact cells including the \(\beta\)-adrenergic and \(\mu\) and \(\delta\) opioid receptors (306-309). Here I used this approach to probe 5-HT\(_{2A}\) phosphorylation in whole cells by immunopurifying wild-type and 5-HT\(_{2A}\)-S314A receptors from \([^{32}P]i\) labeled fibroblasts following EGF activation of RSK2 (240,244). Both the wild-type 5-HT\(_{2A}\) and 5-HT\(_{2A}\)-S314A polyclonal cell lines bound \(^3\)H-ketanserin with \(K_d\) values consistent with reported affinities for the 5-HT\(_{2A}\) receptor (wild-type 5-HT\(_{2A}\) \(K_d=3.08 +/- 0.45\) nM, N=3; 5-HT\(_{2A}\)-S314A \(K_d=3.18 +/- 0.50\) nM, N=4) and expressed similar amounts of receptor (wild-type 5-HT\(_{2A}\) \(B_{max} = 703 +/- 190\) fmol/mg protein, 5-HT\(_{2A}\)-S314A \(B_{max} = 818 +/- 106\) fmol/mg protein). As shown in Figure 3-8C, EGF time-dependently activated endogenous RSK2 in these fibroblasts with maximal activation occurring at 10 min. Autoradiographic analysis of receptors immunopurified from EGF-activated, \([^{32}P]i\)-labeled fibroblasts detected receptor phosphorylation in the wild-type 5-HT\(_{2A}\) fibroblasts, but failed to detect phosphorylation in 5-HT\(_{2A}\)-S314A fibroblasts and control fibroblasts lacking the tagged receptor (arrow at ~62 kDa, Figure 3-8D). These results, taken together with the previous \textit{in vitro} data, show that activated RSK2 specifically
phosphorylates Ser314 within the 5-HT$_{2A}$ receptor i3 loop *in vitro* and in intact cells. Furthermore, these data show that growth factor-mediated activation of RSK2 leads to 5-HT$_{2A}$ receptor phosphorylation—a finding that suggests growth-factor activation could result in 5-HT$_{2A}$ desensitization.

**Figure 3-8. Validation of S314 phosphorylation in 5-HT$_{2A}$ receptors in vitro and in MEFs.** For these experiments purified FLAG-His$_6$ 5-HT$_{2A}$ receptors were phosphorylated *in vitro* using purified, activated RSK2 and immunoblotted for phospho-Ser314 (A-B). $[^{32}P]_i$-labeled RSK2+/+ fibroblasts were treated with 0.1ng/mL EGF for various time points and assayed for RSK2 activation (C) and 5-HT$_{2A}$ phosphorylation (D). A, Western blot analysis using the phospho-Ser314 specific antibody (Ser(P)-314 Ab) verified that activated RSK2 phosphorylates Ser314 within the 5-HT$_{2A}$ receptor i3 loop *in vitro* (top panel, arrow at ~62 kDa). The specific RSK inhibitor SL0101 (10 μM) attenuated Ser(P)-314 Ab
immunolabeling. Western blot analysis of the samples loaded for Ser(P)-314 Ab detection confirmed the components of each in vitro kinase assay (lower panels). B, Quantification of 5-HT$_{2A}$ receptor phosphorylation shown in A. The sum pixel intensity values (top, panel A) were normalized to the control assay lacking RSK2 (set to 100%). SL0101 significantly inhibited Ser(P)-314 Ab detection when compared to the in vitro kinase assay without SL0101. Shown here are results from five independent experiments (mean +/- SEM); *$p<0.05$. C, Immunoblots of purified RSK2 were probed with an antibody specific for activated RSK2 (Ser(P)-386) to show that RSK2 was activated following EGF treatment. RSK2 activation was absent in RSK2-/- fibroblasts. D, Wild-type and phosphorylation-deficient 5-HT$_{2A}$-S314A receptors were immunopurified from RSK2+/+ fibroblasts labeled with $[^{32}\text{P}]$ as stated in Chapter 2 MATERIALS AND METHODS. 5-HT$_{2A}$ receptors were not immunopurified from control fibroblasts lacking the FLAG-tagged 5-HT$_{2A}$ receptor. Activation of RSK2 with 0.1 ng/mL EGF resulted in increased $[^{32}\text{P}]$-phosphate incorporation into wild-type 5-HT$_{2A}$, but not 5-HT$_{2A}$-S314A receptors (top panel, arrow ~62 kDa), suggesting that Ser314 is phosphorylated in whole cells. Receptor purifications differed between fibroblast lines (bottom panel) and were normalized for autoradiography.

3.10 RSK2 Requires Ser 314 within the 5-HT$_{2A}$ Receptor I3 Loop to Regulate Agonist Signaling

The results presented here unequivocally show that RSK2 phosphorylates the conserved Ser314 within the i3 loop. To determine if RSK2 requires Ser314
phosphorylation to regulate 5-HT$_2$A receptor signaling I compared the stable fibroblast cell lines previously used for metabolic labeling experiments (wild-type 5-HT$_2$A and phosphorylation-deficient 5-HT$_2$A-S314A) at multiple agonist-induced 5-HT$_2$A signaling pathways (i.e. intracellular Ca$^{2+}$ release and IP accumulation). Since RSK2 phosphorylated Ser314 \textit{in vitro} and in intact cells, I predicted that phosphorylation-deficient 5-HT$_2$A-S314A receptor signaling would be insensitive to endogenous RSK2 modulation and should exhibit increased signaling similar to our observations in RSK2/- fibroblasts (Figure 3-2B).

Consistent with this prediction, all three 5-HT$_2$A receptor agonists (i.e. 5-HT, DOI, and \(\alpha\)-methyl5-HT) exhibited increased efficacy and potency in the phosphorylation-deficient 5-HT$_2$A-S314A fibroblasts when compared to the wild-type 5-HT$_2$A fibroblasts (Table 3-2). Specifically, all three 5-HT$_2$A receptor agonists mobilized significantly more intracellular Ca$^{2+}$ in the 5-HT$_2$A-S314A fibroblasts when compared to the wild-type 5-HT$_2$A fibroblasts (Figure 3-9A, Table 3-2). Potencies for all 5-HT$_2$A agonists were significantly increased (2- to 4-fold) in the 5-HT$_2$A-S314A fibroblasts. Similarly, all three 5-HT$_2$A receptor agonists accumulated significantly more IP in the 5-HT$_2$A-S314A fibroblasts when compared to the wild-type 5-HT$_2$A fibroblasts (Figure 3-9B, Table 3-2). Agonist potencies for IP accumulation also significantly increased in 5-HT$_2$A-S314A fibroblasts (2-fold). Since this study used polyclonal stable lines, it is possible that differences in receptor expression between the wild-type 5-HT$_2$A and 5-HT$_2$A-S314A polyclonal stable lines could account for increased 5-HT$_2$A-S314A receptor signaling. Thus I developed a method that simultaneously normalized receptor expression and measured agonist-induced intracellular Ca$^{2+}$ release. I found that at concentrations of
EEDQ that produced equivalent levels of receptor binding in both stable fibroblast lines, stimulation with 5-HT mobilized significantly more intracellular Ca\(^{2+}\) in the 5-HT\(_{2A}\)-S314A fibroblasts when compared to wild-type 5-HT\(_{2A}\) fibroblasts (110 +/- 4.9% vs. 89.8 +/- 2.3% of the untreated maximal response for the 5-HT\(_{2A}\)-S314A and wild-type 5-HT\(_{2A}\) receptors, respectively; \(p<0.05\), N=4, Figure 3-9C). These results are identical to observations in the untreated polyclonal stable lines and suggest that the small difference in receptor expression between cell lines fails to account for the apparent RSK2-insensitivity of the phosphorylation-deficient 5-HT\(_{2A}\)-S314A receptor.

Another possibility is that changes in surface receptor expression due to altered RSK2 regulation account for the 5-HT\(_{2A}\)-S314A signaling phenotype. Previous surface biotinylation data from RSK2+/+ and RSK2-/- fibroblasts demonstrated that significant alterations in surface receptor expression do not occur (172). Taken together, these functional studies demonstrate that removing the Ser314 phosphorylation site within the 5-HT\(_{2A}\) receptor i3 loop renders it resistant to negative regulation by RSK2. Moreover, the potentiated signaling observed with the phosphorylation-deficient 5-HT\(_{2A}\)-S314A receptor is remarkably similar to observations in RSK2-/- fibroblasts and demonstrates that the 5-HT\(_{2A}\)-S314A receptor truly is RSK2-insensitive.
Figure 3-9. Regulation by RSK2 requires S314 phosphorylation within the 5-HT$_{2A}$ receptor i3 loop. RSK2+/+ fibroblasts stably expressing either the wild-type 5-HT$_{2A}$ or phosphorylation-deficient 5-HT$_{2A}$-S314A receptors were treated with 5-HT$_{2A}$ agonists and increases in intracellular Ca$^{2+}$ (A) and inositol phosphates(IP) (B) were measured. Wild-type and 5-HT$_{2A}$-S314A receptor signaling were also examined.
after normalizing receptor expression with EEDQ (C) as outlined in Chapter 2
MATERIALS and METHODS. A, FLIPR analysis using a calcium sensitive dye
shows that 5-HT, DOI, and α-methyl5-HT elicited significantly greater increases in
intracellular Ca^{2+} in 5-HT_{2A}\text{-S314A} fibroblasts relative to wild-type 5-HT_{2A}-
expressing fibroblasts. The baseline-subtracted relative fluorescent unit (RFU)
values were normalized to cell number and maximal wild-type 5-HT response (set to
100\%). Shown here are results from three independent experiments (mean +/-
SEM) in which all measures of relative efficacy (E_{max}) and potency (EC_{50}) were
significant, \( p<0.05 \). B, Scintillation proximity assays show that 5-HT, DOI, and α-
methyl5-HT elicited significantly greater increases in [^{3}\text{H}]\text{-inositol phosphates in 5-}
HT_{2A}\text{-S314A} fibroblasts relative to wild-type 5-HT_{2A}-expressing fibroblasts. The
baseline-subtracted cpm values were normalized to cell number and maximal wild-
type 5-HT response (set to 100\%). Shown here are results from three independent
experiments (mean +/- SEM) in which all measures of relative efficacy (E_{max}) and
potency (EC_{50}) were significant, \( p<0.05 \). C, Under conditions of equal receptor
expression, 5-HT_{2A}\text{-S314A} fibroblasts released more intracellular Ca^{2+} than wild-
type 5-HT_{2A}-expressing fibroblasts. Shown here are normalized relative efficacy
values (maximal untreated wild-type 5-HT response set to 100\% as in (A) from four
independent experiments (mean +/- SEM) in which EEDQ treatments produced
similar levels of specific [^{3}\text{H}]\text{-ketanserin binding; \( ^{*}p<0.05 \).}
Table 3-2

Differences in agonist-mediated signaling between wild-type 5-HT\textsubscript{2A} and phosphorylation-deficient 5-HT\textsubscript{2A}-S314A receptors in RSK2+/+ fibroblasts

<table>
<thead>
<tr>
<th>Cell line/agonist</th>
<th>Ca\textsuperscript{2+} release</th>
<th>IP accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agonist potency (a) EC\textsubscript{50} (pEC\textsubscript{50} ± SEM (b))</td>
<td>Relative agonist efficacy, (%) (c) (E_{\text{max}} +/-) SEM</td>
</tr>
<tr>
<td>Wild-type/5-HT</td>
<td>18.2nM (7.74 ± 0.10)</td>
<td>98.7 ± 3.7</td>
</tr>
<tr>
<td>Wild-type/DOI</td>
<td>8.27nM (8.08 ± 0.26)</td>
<td>61.5 ± 5.6</td>
</tr>
<tr>
<td>Wild-type/\textalpha Me5-HT</td>
<td>29.5nM (7.53 ± 0.11)</td>
<td>95.3 ± 4.1</td>
</tr>
<tr>
<td>S314A/5-HT</td>
<td>3.83nM (8.42 ± 0.08(d))</td>
<td>142 ± 3(d)</td>
</tr>
<tr>
<td>S314A/DOI</td>
<td>2.95nM (8.53 ± 0.12(d))</td>
<td>130 ± 5(d)</td>
</tr>
<tr>
<td>S314A/\textalpha Me5-HT</td>
<td>6.24nM (8.21 ± 0.07(d))</td>
<td>140 ± 4(d)</td>
</tr>
</tbody>
</table>

\(a\)Agonist potencies (EC\textsubscript{50}) and efficacies (\(E_{\text{max}}\)) were determined for agonist-mediated Ca\textsuperscript{2+} release and inositol phosphate (IP) accumulation. The results represent the average of three independent experiments.

\(b\)pEC\textsubscript{50} values are represented as –log of EC\textsubscript{50} in M.

\(c\)For each experiment the maximum response to 5-HT in fibroblasts expressing wild-type 5-HT\textsubscript{2A} receptors was set equal to 100%.

\(d\)For each agonist the value was statistically different from fibroblasts expressing wild-type 5-HT\textsubscript{2A} receptors, \(p<0.05\).

3.11 Discussion

In this chapter I demonstrated via multiple lines of evidence that RSK2, a downstream effector of ERK that interacts with and negatively regulates 5-HT\textsubscript{2A} receptor signaling in fibroblasts (172), exerts its novel regulatory effect on 5-HT\textsubscript{2A} activity by phosphorylating the i3 loop at the conserved Ser314. This result is important because it is the first successful demonstration of a \textit{bona fide} 5-HT\textsubscript{2A} receptor phosphorylation site and because it is the first evidence that a down-stream member of the ERK/MAPK...
Pathway phosphorylates a GPCR. Additionally, I correlated receptor phosphorylation at Ser314 with altered receptor activity. These findings suggest for the first time that Ser314, and possibly other conserved residues situated in the C-terminal portion of the i3 loop, play a critical role in determining 5-HT$_{2A}$ receptor activity.

Protein phosphorylation, in general, is an indispensable post-translational regulatory mechanism mediated by protein kinases and exploited by the cell to modulate protein signaling cascades, enzyme catalysis, and protein-protein interactions. For GPCRs, protein kinases induce GPCR desensitization via a process in which distinct intracellular residues are phosphorylated, thereby uncoupling the receptor from its cognate G protein (103). For more than 20 years it has been appreciated that various kinases can regulate 5-HT$_{2A}$ signaling (182,215), and although it is clear that PKC plays an important role in modulating 5-HT$_{2A}$ receptor signaling, it is unclear if PKC or any other kinase directly phosphorylates the 5-HT$_{2A}$ receptor. A recent study by Turner and Raymond (161) using purified PKC and a peptide corresponding to amino acids 377-396 of the human 5-HT$_{2A}$ receptor C-terminus provided the first direct biochemical evidence that PKC can phosphorylate a 5-HT$_{2A}$-derived peptide in vitro. Moreover, PKC-mediated phosphorylation of the peptide was inhibited by calmodulin thereby providing an intriguing regulatory scenario (188). A previous study by Vouret-Craviari et al. (190) identified basal but not agonist-induced phosphorylation of the 5-HT$_{2A}$ receptor in HEK293 cells, although the identity of the kinase was not determined. Despite the ability of PKC, and most likely other kinases, to modulate 5-HT$_{2A}$ signaling it was still unclear, until now, whether 1) the full-length 5-HT$_{2A}$ receptor is phosphorylated by a specific kinase(s) and 2) if 5-HT$_{2A}$ receptor phosphorylation itself modulates receptor signaling.
Work by Sheffler et al. (172) showed that RSK2 negatively regulates 5-HT$_{2A}$ signaling, as evidenced by augmented transient Ca$^{2+}$ release and IP accumulation in RSK2/- fibroblasts. Here I provide supporting time course data which show that both rapid and prolonged 5-HT$_{2A}$ signaling events are dysregulated in RSK2 -/- fibroblasts. Close examination of previous results suggested that RSK2 most likely negatively regulates 5-HT$_{2A}$ receptor function proximal to receptor activation by directly phosphorylating the receptor i3 loop. Specifically, I decided to focus on receptor phosphorylation since previous evidence indicated that RSK2 interacts with the 5-HT$_{2A}$ receptor i3 loop within a highly conserved region containing the RSK2-like phosphorylation motif $^{275}$R-A-K-L-A-$^{528}$S, and extensive control experiments suggested that signaling components downstream from the receptor are unaltered.

In the approach outlined here I incorporated a variety of techniques designed to dissect and unequivocally determine if RSK2 modulates 5-HT$_{2A}$ function via direct receptor phosphorylation. The data presented here progressed from 1) initial experiments which demonstrated that RSK2 kinase activity is essential for attenuating 5-HT$_{2A}$ signaling and promoting 5-HT$_{2A}$ receptor phosphorylation (i.e. kinase-dead rescue assays and in vitro kinase assays), to 2) experiments that identified and validated the novel Ser314 phosphorylation site (i.e. tandem MS, site-directed mutagenesis, phospho-specific immunoblotting, and metabolic labeling assays), to 3) functional experiments which determined that removal of the phospho-acceptor site (S314A) compromises RSK2’s ability to negatively regulate 5-HT$_{2A}$ signaling (i.e. intracellular Ca$^{2+}$ release and IP accumulation assays). In this approach I incorporated a broad spectrum of standard
techniques such as MS and [\(^{32}\)P] metabolic labeling that have been invaluable for the
discovery of GPCR phosphorylation sites (111,306-312).

One particularly surprising result of these experiments was that the unbiased MS
experiments failed to identify Ser280 as a major site of RSK2 phosphorylation in both the
i3 peptide and full-length receptor. I initially predicted that RSK2 would phosphorylate
Ser280 located within the RSK2-like consensus phosphorylation motif \(^{275}\)R-A-K-L-A-S\(^{280}\), a region of the i3 loop which is highly conserved among rat, human, and mouse 5-HT\(_{2A}\) receptors. The failure to demonstrate that RSK2 targets Ser280 within the
consensus phosphorylation motif was independently verified in the i3 loop peptide using
both site-directed mutagenesis and phospho-specific immunoblotting strategies. Evidence
showing that the highly conserved RSK2-like \(^{275}\)R-A-K-L-A-S\(^{280}\) phosphorylation motif
did not contribute to receptor phosphorylation suggests that the motif may have
additional functions. For example, it is likely that this region targets RSK2 to the 5-HT\(_{2A}\)
receptor, since yeast-two-hybrid experiments showed that this region of the i3 loop
facilitates RSK2 binding (172). Several low abundance phosphopeptides were also
identified, but similar to Ser280, site-directed mutagenesis of a subset of these sites failed
to influence overall phosphorylation levels. It is important to keep in mind that even
though i3 loop phosphorylation was almost completely attenuated using the S314A
mutant, it is possible that these sites contribute to some aspect of 5-HT\(_{2A}\) receptor
regulation. This caveat is supported by studies showing that intracellular regions of
GPCRs can be multiply phosphorylated and represents a flexible regulatory mechanism
that can be tailored in a tissue-specific manner to regulate physiological processes (181).
Importantly, almost complete loss of [\(^{32}\)P] incorporation into the phosphorylation-
deficient 5-HT<sub>2A</sub>-S314A receptor in the metabolic labeling experiment suggested that few, if any, additional RSK2 phosphorylation sites exist. However, the possibility of sequential phosphorylation cannot be ruled out. These results strongly suggest that RSK2 favors Ser314 phosphorylation *in vitro* and in intact cells.

The finding that RSK2 phosphorylation within a conserved region of the i3 loop has functional consequences is consistent with several studies by our laboratory and others suggesting that the i3 loop is important for directing and promoting GPCR signaling. Specifically, we demonstrated that the purified 5-HT<sub>2A</sub> receptor i3 loop is predominantly α-helical and binds purified arrestins (199). Previous work also suggests that peptides corresponding to C-terminal regions of the 5-HT<sub>2A</sub> i3 loop directly interact with and activate purified Gαq (123). These findings agree with several studies suggesting that amphipathic α-helices are involved in determining receptor-G protein binding (313,314), and may, in fact, be intrinsic to Gαq coupling as suggested by the recent structure of Gαq-coupled squid rhodopsin (25). Furthermore, merely introducing the i3 loop of the Gαq-coupled 5-HT<sub>2A</sub> receptor into the Gαi-coupled 5-HT<sub>1B</sub> receptor (5-HT<sub>1B/2A</sub>) is sufficient to shift its coupling specificity from inhibition of adenylyl cyclase to PLCβ activation (315). Together with previous studies showing that introduction of phosphorylated residues into the cytoplasmic domains of many GPCRs inhibits signaling, it seems likely that Ser314 phosphorylation functionally uncouples the 5-HT<sub>2A</sub> receptor from its cognate G protein.

An additional mechanism whereby Ser314 phosphorylation (denoted here as 6.26 (37), arrow **Figure 3-7B**) modulates 5-HT<sub>2A</sub> receptor function could be related to its close proximity to a highly conserved salt bridge between Glu318 on transmembrane
helix VI (E318, denoted 6.30, arrow **Figure 3-7B**) and an Arg residue (denoted 3.50) located in a highly conserved stretch of amino acids (D/E-R-Y) located at the interface between transmembrane III and the second intracellular loop (36). This intramolecular salt bridge interaction is thought to play a pivotal role in regulating GPCR conformational states since it is retained in the ground state structure of rhodopsin and disrupted in the constitutively active β2AR (23,30). Specifically, homology modeling of the 5-HT2A receptor based on rhodopsin places Ser314 (6.26) one helical turn down from Glu318 (6.30) and studies from our laboratory show that disrupting this ‘ionic lock’ by mutating Glu318 (6.30) to Arg increases constitutive activity and agonist affinity of the 5-HT2A receptor (35). With regard to the data in this chapter, it remains a possibility that the converse situation also occurs; that phosphorylation of Ser314 (6.26) one helical turn down from Glu318 (6.30) promotes stability of the ionic lock, thus favoring the inactive receptor conformation. In fact, preliminary evidence suggests that either of these scenarios is plausible since mutating Ser314 to Asp (i.e. phosphomimetic) in the 5-HT2A receptor attenuates agonist-induced IP accumulation (Gray and Roth, unpublished observations). Nonetheless, the mechanism explaining how Ser314 (6.26) phosphorylation negatively regulates 5-HT2A receptor function is fascinating and will be the subject of intense future investigation.

Most importantly, these findings are the first to conclusively demonstrate that a downstream MAPK/ERK cascade signaling protein interacts with and directly phosphorylates a GPCR, thereby modulating its signaling capacity. Therefore, our results, together with a large body of literature showing that GPCRs activate the ERK/MAPK cascade via numerous mechanisms, are consistent with the intriguing possibility that
RSK2 is activated downstream of ERK, thereby exerting negative feedback control on 5-HT$_{2A}$ signaling (i.e. homologous desensitization). To address this, I initially attempted to detect receptor phosphorylation in RSK2+/- and RSK2-/- MEFs following 5-HT stimulation via metabolic labeling experiments. In contrast to the ability of activated RSK2 to phosphorylate the receptor in intact cells (Figure 3-8D), I failed to detect agonist-induced 5-HT$_{2A}$ phosphorylation. This finding was not surprising considering that previous attempts also failed to detect agonist-induced 5-HT$_{2A}$ phosphorylation (172,190,191). It remains a possibility that the method used here was not sensitive enough to detect low levels of receptor phosphorylation; therefore, future studies will try to determine if RSK2 plays an important role in mediating homologous desensitization of the 5-HT$_{2A}$ receptor.

The data presented in Chapter 3 could also be explained by an alternative mechanism. Specifically, the metabolic labeling data are consistent with a heterologous desensitization model wherein growth factors activate RTKs and downstream MAPKs, which in turn activate RSK2 and phosphorylate the 5-HT$_{2A}$ receptor to modulate its function. This mechanism has been recently described for only a few GPCRs (i.e. $\alpha_{1b}$AR, $\beta_1$AR, $\beta_2$AR, and 5-HT$_{2C}$ receptor) and will be explored in Chapter 4.
CHAPTER 4: RECEPTOR TYROSINE KINASE (RTK)-GPCR CROSS-TALK: EVIDENCE FOR A MECHANISM WHEREBY RTKs ACT VIA RSK2 TO MODULATE GPCR SIGNALING

4.1 Introduction and Rationale

As shown in Chapter 3, I demonstrated via multiple, independent lines of evidence that RSK2, a multifunctional effector of the ERK/MAPK cascade, is a novel GPCR kinase. Specifically, RSK2 interacts with and directly phosphorylates the 5-HT$_{2A}$ receptor at Ser314, thereby modulating its signaling. Moreover, autoradiographic analysis of receptors immunopurified from EGF-activated, [$^{32}$P]$_i$-labeled MEFs showed that growth-factor activated RSK2 phosphorylates wild-type 5-HT$_{2A}$ receptors, but not S314A 5-HT$_{2A}$ receptors, in live cells. These data were extremely exciting since they 1) provided in situ support of the previous mass spectrometry, site-directed mutagenesis, and phospho-specific antibody data implicating Ser314 as the RSK2 phospho-acceptor site and 2) suggested that EGF activation could, in theory, lead to 5-HT$_{2A}$ receptor desensitization given the functional effects of Ser314 phosphorylation.

In fact, correlating the EGF-mediated activation of RSK2 with the potential for 5-HT$_{2A}$ desensitization is not entirely unfounded since recent reports have shown that considerable bi-directional cross-talk occurs between GPCRs and receptor tyrosine kinases (RTKs). First, GPCR signaling through an entirely G protein-mediated pathway fails to account for the diversity of responses resulting from GPCR activation, especially effects on cell growth and differentiation. Thus, it is now realized that these responses are mediated by complex GPCR signaling networks that converge upon the activation of
MAPKs (78). It is typically thought that GPCRs activate the MAPK cascade through numerous effectors including transactivation of EGF, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and neurotrophin RTKs. Specifically, GPCRs are known to transactivate RTKs via several routes including a ligand-dependent process utilizing ADAM family metalloproteases resulting in subsequent ectodomain shedding of RTK ligands, as well as ligand-independent processes including activation of non-receptor tyrosine kinases and production of reactive oxygen species (ROS). In fact, recent studies have identified several signaling intermediates linking the 5-HT$_{2A}$ receptor to ERK/MAPK transactivation. In vascular smooth muscle cells, 5-HT$_{2A}$ activates ERK via transactivation of receptor tyrosine kinases (173) and activation of MEK1 (175), and NHE1 (176). In addition, several detailed studies have identified signaling intermediates linking the 5-HT$_{2A}$ receptor to ERK/MAPK activation. Greene et al. (177) reported that 5-HT$_{2A}$ receptors in rat renal mesangial cells transactivate ERK via H$_2$O$_2$ generation. Specifically a signaling pathway progressing from PLC and PKC activation to NAD(P)H oxidase-mediated generation of ROS were implicated in the activation of ERK. An additional study in renal mesangial cells found that tumor necrosis factor $\alpha$-converting enzyme (TACE, ADAM17) was responsible for 5-HT$_{2A}$-mediated, ligand-induced transactivation of the EGF receptor and ultimately activation of ERK (178). These studies definitively show that 5-HT$_{2A}$ receptors activate ERK/MAPK, thereby accounting for 5-HT$_{2A}$-mediated vascular smooth muscle contraction (173), cellular proliferation (174) and the induction of the early response genes cyclo-oxygenase-2 and Egr-1 (165).

Second, it is likely that EGF-mediated activation promotes 5-HT$_{2A}$ receptor desensitization since recent data support a novel regulatory paradigm whereby RTK
activation modulates GPCR signaling. Specifically, RTK activation profoundly affects the signaling of the β1-, β2-, and α1β-adrenergic receptors (108-112), as well as 5-HT2C receptors (113). Thus, given the important physiological implications behind RTK regulation of GPCR signaling as demonstrated for insulin receptor-mediated inhibition of β2AR signaling (114), it is likely that additional GPCRs are regulated by RTKs. To test the hypothesis that RSK2 activation downstream of growth factor activation of RTKs does, indeed, desensitize 5-HT2A receptor signaling, I applied a battery of experiments incorporating various RTK ligands (i.e. EGF, TGF-α, and PDGF), cell lines (i.e. RSK2+/+ and RSK2−/− MEFs and mVSMCs), and GPCR ligands (i.e. 5-HT, lisuride, 5-methoxyDMT, and ATP). Data from each experiment will be presented in detail below.

4.2 Treatment with Epidermal Growth Factor Time-Dependently Decreases 5-HT-Induced Intracellular Ca2+ Release

The data in Chapter 3 show that RSK2-mediated phosphorylation of the 5-HT2A receptor leads to decreased agonist responsiveness. Moreover, the metabolic labeling data in intact fibroblasts show that EGF-activated RSK2 phosphorylates the 5-HT2A receptor. Thus, it was possible that EGF pretreatment could decrease 5-HT2A receptor responsiveness. To test this possibility, I treated serum-starved RSK2 +/+ and RSK2 −/− fibroblasts with 100 ng/mL EGF for 1 hr and assessed 5-HT-induced Ca2+ release. As shown in Figure 4-1A, RSK2 was maximally activated 10 min after treatment with 100 ng/mL human EGF (hEGF). Interestingly, treatment of MEFs stably expressing FLAG-5HT2A receptors with hEGF shifted 5-HT concentration-response curves to the right in a time-dependent manner. A maximal effect was obtained at 1 hr (Figure 4-1B), as longer
time points (i.e. 2-4 hr) did not lead to increased shifts in potency (Figure 4-1C). Specifically, 1 hr hEGF treatment caused a significant 4-fold decrease in potency for the full agonist 5-HT (3.5 nM vs. 15 nM for untreated and hEGF-treated, respectively; N=10 independent experiments, \( p<0.05 \)) and was indicative of receptor desensitization in an over-expressed experimental system. It is important to note that extensive serum-starvation was required (24-48hr) in order to see this effect, since initial studies showed that 6 hr of serum starvation was inadequate. It is well known that serum contains multiple growth factors including EGF, leading to desensitization of growth-factor-mediated responses. Thus, serum starvation is routinely employed to investigate growth-factor mediated responses and pathways. Additionally, serum contains micromolar concentrations of 5-HT which also leads to considerable desensitization of serotonergic signaling pathways.

Since rightward shifts in 5-HT potency were observed following hEGF treatment, I plotted the \( \text{Ca}^{2+} \) traces obtained with 10 nM 5-HT (\(~\text{EC}_{50}\) ) to observe any effects on \( \text{Ca}^{2+} \) release. As expected, hEGF treatment time-dependently decreased the peak 5-HT-induced \( \text{Ca}^{2+} \) release, with a maximal effect at 1 hr (Figure 4-1D). Again, I did not observe significant decreases in peak \( \text{Ca}^{2+} \) release at the 2-4 hr time points (Figure 4-1E). These results show that 5-HT\(_{2A}\) receptor responsiveness to the endogenous agonist is attenuated by hEGF pre-treatment. Moreover, these results represent the first evidence suggesting that growth factors facilitate heterologous desensitization of a 5-HT receptor. The physiological implications of this regulation in the brain are tremendous, therefore the rest of Chapter 4 is dedicated to exploring the mechanism whereby growth factors
desensitize the 5-HT$_{2A}$ receptor, as well as its application to other GPCRs previously shown to be regulated by RSK2 (172).
Figure 4-1. Human EGF pre-treatment desensitizes 5-HT\textsubscript{2A} receptor signaling in RSK2\textsuperscript{+/-} MEFs. RSK2\textsuperscript{+/-} mouse embryonic fibroblasts (MEFs) stably expressing FLAG 5-HT\textsubscript{2A} receptors were treated as detailed in Chapter 2 MATERIALS AND METHODS. Briefly, RSK2\textsuperscript{+/-} MEFs were pre-treated with 100 ng/mL human EGF (hEGF) for various time points and RSK2 activation (A) and 5-HT-induced intracellular Ca\textsuperscript{2+} release (B-D) were determined. A, Immunoblots of purified RSK2 were probed with an antibody specific for activated RSK2 (Ser(P)-386 Ab) to show that RSK2 was robustly activated following EGF treatment. RSK2 activation was absent in RSK2\textsuperscript{-/-} MEFs. B, 5-HT concentration-response curves for Ca\textsuperscript{2+} release in serum-starved RSK2\textsuperscript{+/-} MEFs following 0 min (■), 30 min (○), and 1 hr (▲) hEGF pre-treatment show time-dependent attenuation of Ca\textsuperscript{2+} response. Specifically, rightward shifts in 5-HT potency are significantly different at the 30 and 1 hr times; N=3-10 independent experiments (mean +/- SEM), \( p<0.05 \). No effects on maximal efficacy were detected. C, 5-HT concentration-response curves for Ca\textsuperscript{2+} release in serum-starved RSK2\textsuperscript{+/-} MEFs following 0 hr (■), 2 hr (○), and 4 hr (▲) hEGF pre-treatment show no significant decreases in 5-HT response; N=2 independent experiments (mean +/- SEM), \( p>0.05 \). D, Normalized FLIPR Ca\textsuperscript{2+} traces (maximal Ca\textsuperscript{2+} response in untreated MEFs was set to 100\%) induced by 10 nM 5-HT illustrate the time-dependent decrease in Ca\textsuperscript{2+} response following 0 min (■), 30 min (○), and 1 hr (▲) hEGF pre-treatment. All time points were significantly different from the peak Ca\textsuperscript{2+} response in untreated MEFs; N=3 independent experiments (mean +/- SEM), \( p<0.05 \). E, hEGF pre-treatment for 0 hr (■), 2 hr (○), and 4 hr (▲)
failed to diminish the maximal Ca$^{2+}$ response compared to untreated MEFs; N=2 independent experiments (mean +/- SEM).

4.3 Treatment with Epidermal Growth Factor Decreases 5-HT-Induced Intracellular Ca$^{2+}$ Release in a Concentration-Dependent Manner

Previous results showed that pre-treating RSK2+/+ MEFs stably expressing FLAG 5-HT$_{2A}$ receptors for 1 hr with 100 ng/mL hEGF decreased 5-HT$_{2A}$ receptor responsiveness. Therefore, in these experiments my goal was to further characterize and verify this effect by calculating an IC$_{50}$ for hEGF. As detailed in Chapter 2 MATERIALS AND METHODS, I pre-treated MEFs for 1 hr with varying concentrations of hEGF (10 pg/mL to 10 μg/mL) and then generated full 5-HT concentration-response curves for Ca$^{2+}$ release at each concentration of hEGF. I then calculated an IC$_{50}$ for hEGF by plotting pEC$_{50}$ values for 5-HT vs. hEGF concentration. As shown in Figure 4-2A, 5-HT concentration-response curves were shifted to the right in a concentration-dependent manner, consistent with my previous results using a fixed concentration of hEGF (100 ng/mL). Plotting pEC$_{50}$ vs. hEGF concentration yielded an IC$_{50}$ of 3.0 ng/mL (Figure 4-2B), consistent with reported values for hEGF-mediated tyrosine phosphorylation of EGF receptor (i.e. 7.2 ng/mL) (316). These results suggest that hEGF inhibits 5-HT$_{2A}$ signaling within a concentration range that corresponds with physiological EGF receptor activation and supports the data in Chapter 4.2. Moreover, the concentration-dependence of these data suggest that this novel mechanism cannot be explained by non-specific effects such as those associated with a single large dose of hEGF.
Figure 4-2. hEGF desensitizes 5-HT$_{2A}$ receptor signaling with a potency consistent with its ability to activate the EGF receptor. A, The potency with which hEGF desensitizes 5-HT$_{2A}$-mediated Ca$^{2+}$ release (IC$_{50}$) was calculated by generating 5-HT concentration-response curves for Ca$^{2+}$ release after pre-treating MEFs for 1 hr with varying concentrations of hEGF (10 pg/mL to 10 μg/mL). As expected, hEGF pre-treatment dose-dependently shifted concentration-response curves to the right, thereby decreasing 5-HT potency. These data are from four independent experiments (mean +/- SEM). B, Plotting the pEC$_{50}$ values derived from 5-HT concentration-response curves vs. hEGF concentration generated an IC$_{50}$ of 3.0 ng/mL for hEGF. This value agrees with the ability for hEGF to activate the EGF receptor (i.e. 7.2 ng/mL).
4.4 RSK2 is Required for hEGF-Mediated Heterologous Desensitization of 5-HT$_{2A}$ Receptor Signaling

From the previous experiment it is clear that EGF receptor activation leads to decreased 5-HT$_{2A}$ responsiveness in MEFs; however, the role of RSK2 remains unclear. In order to directly test the hypothesis that growth factor-activated RTKs desensitize 5-HT$_{2A}$ receptor signaling via RSK2 activation, I pre-treated RSK2-/- MEFs stably expressing FLAG 5-HT$_{2A}$ receptors with hEGF as detailed in Chapter 4.2. Importantly, the RSK2-/- MEFs were treated in parallel with RSK2+/+ MEFs so direct comparisons could be made between experiments in which EGF receptor activation decreased 5-HT$_{2A}$ signaling.

In agreement with my initial hypothesis, I discovered that RSK2 was required for hEGF-induced heterologous desensitization of 5-HT$_{2A}$ receptors. Specifically, I failed to detect any shifts in potency for 5-HT concentration-response curves at any of the times tested (0-4 hr) (Figure 4-3A). In fact, 1 hr pre-treatment with hEGF, which produced a maximal effect in RSK2+/+ MEFs, produced nearly identical potencies in RSK2-/- MEFs (24 nM vs. 17 nM in untreated and hEGF-treated MEFs, respectively; p>0.05). Interestingly, prolonged hEGF treatment (2-4 hr) did not affect 5-HT potency but trended towards increased 5-HT maximal efficacy (E$_{\text{max}}$) (Figure 4-3B). This potentiation was not observed in RSK2+/+ MEFs and is both intriguing and puzzling considering its rapid induction, occurring as early as 2 hr and likely indicates some undefined counter-regulatory process.

Inspection of normalized peak Ca$^{2+}$ responses elicited by an EC$_{50}$ concentration of 5-HT showed that hEGF pre-treatment failed to significantly alter the responsiveness of
5-HT$_{2A}$ receptors (Figure 4-3C and 3D). These results are opposite to what I observed with the RSK2+/+ MEFs and suggest for the first time that RSK2 is necessary for RTK-mediated heterologous desensitization of a GPCR.

![Graphs showing Ca$^{2+}$ response](image)

Figure 4-3. RSK2 is required for hEGF-mediated heterologous desensitization of 5-HT$_{2A}$ receptors. RSK2/- mouse embryonic fibroblasts (MEFs) stably expressing FLAG 5-HT$_{2A}$ receptors were treated and the data analyzed as detailed in Chapter 2 MATERIALS AND METHODS. Briefly, RSK2/- MEFs were pre-treated with
100ng/mL human EGF (hEGF) for various times and 5-HT-induced intracellular Ca\(^{2+}\) release was subsequently determined. A, 5-HT concentration-response curves for Ca\(^{2+}\) release in serum-starved RSK2/-/ MEFs following 0 min (■), 30 min (○), and 1 hr (▲) hEGF pre-treatment show that hEGF fails to decrease 5-HT potency in RSK/-/ MEFs; N=3-6 independent experiments (mean +/- SEM), p>0.05. No effects on maximal efficacy were detected. B, 5-HT concentration-response curves for Ca\(^{2+}\) release in serum-starved RSK2/-/ MEFs following 0 hr (■), 2 hr (○), and 4 hr (▲) hEGF pre-treatment show no significant decreases in 5-HT response; N=2 independent experiments (mean +/- SEM), p>0.05. C, Normalized FLIPR Ca\(^{2+}\) traces (maximal Ca\(^{2+}\) response in untreated MEFs was set to 100%) induced by 10 nM 5-HT show no significant decrease in Ca\(^{2+}\) response following 0 min (■), 30 min (○), and 1 hr (▲) hEGF pre-treatment; N=3 independent experiments (mean +/- SEM), p<0.05. D, Normalized FLIPR Ca\(^{2+}\) traces (maximal Ca\(^{2+}\) response in untreated MEFs was set to 100%) induced by 10 nM 5-HT showed that hEGF pre-treatment for 0 hr (■), 2 hr (○), and 4 hr (▲) failed to diminish the maximal Ca\(^{2+}\) response compared to untreated MEFs; N=2 independent experiments (mean +/- SEM).
4.5 RSK2 is Required for TGF-α-Mediated Heterologous Desensitization of 5-HT$_{2A}$ Receptor Signaling

In an effort to validate that the EGF receptor was, indeed, responsible for RTK-mediated heterologous desensitization of 5-HT$_{2A}$ receptors in MEFs, as well as further explore the requirement for RSK2, I tested an additional EGF receptor ligand. Of the seven known mammalian EGF receptor ligands (i.e. EGF, transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor, amphiregulin, betacellulin, epiregulin, and epigen), I chose to study the effects of TGF-α since both EGF and TGF-α are expressed within the same brain regions (317). These experiments were performed and analyzed identically as described in Chapter 2 MATERIALS AND METHODS, except that the cells were treated with TGF-α for only 1 hr. Importantly, I used the 1 hr pre-treatment since it maximally desensitized 5-HT$_{2A}$ receptors in RSK2+/+ MEFs but had no effect in RSK2/- MEFs. As shown in Figure 4-4A, TGF-α pre-treatment produced rightward shifts in 5-HT concentration-response curves, resulting in a significant 2.5-fold reduction in potency (2.2 nM vs. 5.6 nM for untreated and hTGF-α-treated MEFs, respectively; $p<0.05$). These data are consistent with desensitization of the full agonist 5-HT and support results obtained using hEGF. Also in support of the hEGF data, I determined that TGF-α decreased 5-HT potency with an IC$_{50}$ of 2.7 ng/mL (Figure 4-4B). As with hEGF, this novel regulatory mechanism requires the activation of EGF receptors within a concentration range commonly observed for typical EGF receptor-mediated events. Taken together, these data demonstrate that EGF receptor activation causes time- and concentration-dependent desensitization of 5-HT$_{2A}$ receptor responsiveness.
I also observed that TGF-α treatment produced a small but significant decrease in maximal efficacy ($E_{\text{max}}$) ($E_{\text{max}}=100\%$ vs. $93\%$ in untreated and hTGF-α-treated MEFs, respectively; $p<0.05$). Although this effect is statistically significant it is likely of no physiological significance.

In contrast to the effects in RSK2+/+ MEFs, TGF-α pre-treatment failed to induce dextral shifts in 5-HT concentration-response curves in RSK2/- MEFs. As shown in Figure 4-4C, the 5-HT concentration-response curve for untreated RSK2/- MEFs was indistinguishable from the TGF-α-treated curve. These data are similar to those obtained using hEGF and support the hypothesis that RSK2 is required for RTK-mediated heterologous desensitization of 5-HT$_{2A}$ receptors.
Figure 4-4. RSK2 is required for TGF-α-mediated heterologous desensitization of 5-HT$_{2A}$ receptors. RSK2+/+ and RSK2−/− mouse embryonic fibroblasts (MEFs) stably expressing FLAG 5-HT$_{2A}$ receptors were treated as detailed in Chapter 2 MATERIALS AND METHODS. Briefly, MEFs were pre-treated with 100 ng/mL TGF-α for various times and 5-HT-induced intracellular Ca$^{2+}$ release was determined. A, 5-HT concentration-response curves for Ca$^{2+}$ release in serum-starved RSK2+/+ MEFs following 0 min (■), 30 min (○), and 1 hr (▲) TGF-α pre-treatment show time-dependent attenuation of Ca$^{2+}$ response. Specifically, rightward shifts in 5-HT potency are significantly different at the 30 min and 1 hr time points; N=4 independent experiments (mean +/- SEM), p<0.05. B, The potency with which TGF-α desensitizes 5-HT$_{2A}$-mediated Ca$^{2+}$ release (IC$_{50}$) was calculated by generating 5-HT concentration-response curves for Ca$^{2+}$ release after pre-treating MEFs for 1hr with varying concentrations of TGF-α (0.1 ng/mL to 10 μg/mL). As expected, TGF-α pre-treatment shifted concentration-response curves to the right. Plotting the pEC$_{50}$ values derived from 5-HT concentration-response curves vs. TGF-α concentration generated an IC$_{50}$ of 2.7 ng/mL for hEGF. N=2 independent experiments. C, 5-HT concentration-response curves for Ca$^{2+}$ release in serum-starved RSK2−/− MEFs following 0 (■), 30 (○), and 1 hr (▲)TGF-α pre-treatment show that TGF-α fails to decrease 5-HT potency in RSK−/− MEFs; N=2 independent experiments (mean +/- SEM), p>0.05.
4.6 RSK2 is Required for hEGF-Mediated Heterologous Desensitization of 5-HT$_{2A}$ Receptors-Supporting Data from a Panel of 5-HT$_{2A}$ Agonists with Different Intrinsic Efficacies

The previous results showed that hEGF pre-treatment promotes dextral shifts in 5-HT concentration-response curves (i.e. decreased potency), without a concomitant decrease in maximal efficacy ($E_{max}$). This behavior can be explained according to classic receptor theory in that it is suggestive of desensitization of a full agonist in an over-expressed system (i.e. receptor reserve). This is indeed the case since I am using MEFs stably expressing 5-HT$_{2A}$ receptors at ~2000-4000 fmol/mg. According to classical receptor pharmacology, the maximal efficacy but not potency of full agonists is resistant to decreases in the number of functional receptors resulting from irreversible receptor alkylation (i.e. EEDQ), degradation (i.e. down-regulation), or through a rapid loss of receptor responsiveness (i.e. receptor desensitization). Partial agonists, on the other hand, are less resistant to decreases in the population of active receptors and exhibit characteristic decreases in both potency and maximal efficacy. This behavior is explained by the fact that full agonists require very little receptor occupancy to elicit a full response (large receptor reserve); while partial agonists require more receptor occupancy to signal maximally (low receptor reserve). If this reasoning is correct, then assessing RTK-mediated desensitization of 5-HT$_{2A}$ receptors with the lower intrinsic efficacy agonists (i.e. strong and weak partial agonists), as opposed to the full agonist 5-HT, should reveal significant decreases in both potency ($EC_{50}$) and efficacy ($E_{max}$). In this approach I have essentially forced an over-expressed system to behave as if the receptors were expressed closer to endogenous levels.
Here I used 5-methoxyDMT and lisuride which are both shown in **Chapter 5** to specifically activate 5-HT$_{2A}$ receptors with relative $E_{max}$ values for Ca$^{2+}$ release of 80% and 30%, respectively (5-HT set at 100%). As shown previously, agonist concentration-response curves were generated from serum-starved RSK2+/+ and RSK2-/- MEFs pre-treated for 1 hr with 100 ng/mL hEGF. As expected, the concentration-response curve for 5-methoxyDMT was shifted rightward and downward indicating decreases in both potency and maximal efficacy (**Figure 4-5A**). Specifically, 1 hr hEGF pre-treatment with hEGF significantly decreased the potency of 5-methoxyDMT 3.5-fold ($EC_{50}= 17$ nM vs. 60 nM for untreated and hEGF-treated RSK2+/+ MEFs, respectively; N=2, $p<0.05$). Also as predicted, 5-methoxyDMT relative maximal efficacy was significantly decreased ($E_{max}=88\%$ vs. 77$\%$ for untreated and hEGF-pretreated RSK2+/+ MEFs, respectively; N=2, $p<0.05$).

In support of previous data showing a role for RSK2 in RTK-mediated desensitization, hEGF pre-treatment failed to significantly desensitize the 5-methoxyDMT response in RSK2-/- MEFs (**Figure 4-5B**). Specifically, neither potency ($EC_{50}=80$ nM vs. 98 nM for untreated and hEGF-pre-treated, respectively; N=2, $p>0.05$) nor relative maximal efficacy ($E_{max}=79\%$ vs. 76$\%$ for untreated and hEGF-treated, respectively; N=2, $p>0.05$) were significantly affected in these cells.

The behavior of the weak partial agonist lisuride was also examined in these experiments. Similar to observations made with the strong partial agonist 5-methoxyDMT, both the potency ($EC_{50}= 241$ nM vs. 468 nM for untreated and hEGF-treated RSK2+/+ MEFs, respectively; N=2, $p<0.05$) and maximal efficacy ($E_{max}= 58.5\%$ vs. 40.3$\%$ for untreated and hEGF-treated RSK2+/+ MEFs, respectively; N=2, $p<0.05$)
of lisuride were significantly decreased by hEGF (Figure 4-5C). However, these effects were completely absent in RSK2-/- MEFs (Figure 4-5D). Taken together, these studies involving partial agonists support the requirement for RSK2 in RTK-mediated heterologous desensitization. Moreover, the ability to predict agonist behavior according to receptor theory strongly supports the hypothesis that RTK activation desensitizes 5-HT$_{2A}$ receptors.

**Figure 4-5.** RSK2 is required for RTK-mediated desensitization of 5-HT$_{2A}$ receptor partial agonists. RSK2+/+ and RSK2-/- mouse embryonic fibroblasts (MEFs) stably expressing FLAG 5-HT$_{2A}$ receptors were treated as detailed in Chapter 2.
MATERIALS AND METHODS. Briefly, serum-starved MEFs were pre-treated with 100 ng/mL hEGF for 1 hr and then agonist concentration-response curves for Ca$^{2+}$ were generated. A, 5-methoxyDMT concentration-response curves for Ca$^{2+}$ release in serum-starved RSK2+/+ MEFs following 1 hr (o) hEGF pre-treatment show attenuation of Ca$^{2+}$ response. Specifically, significant decreases in 5-methoxyDMT potency and efficacy were observed at 1 hr; N=2 independent experiments (mean +/- SEM), p<0.05. B, Parallel experiments in RSK2/-/- MEFs did not show significant decreases in potency or efficacy; N=2 independent experiments (mean +/- SEM), p<0.05. C, Lisuride concentration-response curves for Ca$^{2+}$ release in serum-starved RSK2+/+ MEFs following 1 hr (o) hEGF pre-treatment show attenuation of Ca$^{2+}$ response. Specifically, significant decreases in lisuride potency and efficacy were observed at 1 hr; N=2 independent experiments (mean +/- SEM), p<0.05. D, Parallel experiments in RSK2/-/- MEFs did not show significant decreases in potency or efficacy; N=2 independent experiments (mean +/- SEM), p<0.05. These results support the requirement of RSK2 in RTK-mediated heterologous desensitization of 5-HT$_{2A}$ receptors. Furthermore the partial agonists behaved as predicted by receptor theory (i.e. decreased E$_{max}$) and further confirms 5-HT$_{2A}$ receptor desensitization.
4.7 RSK2 is Required for Platelet-Derived Growth Factor (PDGF)-Mediated Desensitization of Endogenous 5-HT2A Receptors in Primary Cultures of Mouse Vascular Smooth Muscle Cells (mVSMCs)

Vascular smooth muscle cells (VSMCs) have served as a model system for investigating 5-HT2A signal transduction since they endogenously express 5-HT2A receptors (173). Specifically, 5-HT2A receptor activation in VSMCs leads to release of intracellular Ca\(^{2+}\) from intracellular stores and is detectable via the FLIPR assay (318). Moreover, RSK2 is expressed in VSMCs and has been shown to be important for mediating some aspects of GPCR signaling (319). Thus, VSMCs represent an ideal system whereby I could explore if additional growth factors desensitize 5-HT2A receptors (e.g. platelet-derived growth factor, PDGF).

In this approach I isolated aortic mVSMCs from RSK2+/+ and RSK2-/- mice as detailed in Chapter 2 MATERIALS AND METHODS. I first validated the mVSMC cultures for expression of smooth muscle α-actin, a specific marker for smooth muscle cells. As shown in Figure 4-6A, the isolation technique produced ex vivo cultures that were enriched in vascular smooth muscle cells. Secondly, I validated that I could measure 5-HT2A receptor activation in mVSMCs via FLIPR measurements of intracellular Ca\(^{2+}\). As shown in Figure 4-6B, 5-HT activated endogenous 5-HT2A receptors with a potency of 77 nM. Importantly, I ensured that this Ca\(^{2+}\) response was mediated through 5-HT2A receptors by blocking receptor activation with the 5-HT2A-specific antagonist MDL100907 (Figure 4-6B).
Figure 4-6. Detection of 5-HT$_{2A}$-mediated Ca$^{2+}$ release in mouse vascular smooth muscle cells (mVSMCs). mVSMCs were isolated as described in Chapter 2 MATERIALS AND METHODS. A, Immunofluorescent detection of smooth muscle α-actin (red) verified that the isolation technique produced cultures enriched in mVSMCs. MEFs were used as a negative control and were not immunoreactive. Nuclei were stained with Hoechst (blue). B, 5-HT produced a reliable Ca$^{2+}$ response in mVSMCs. Pre-treatment with the 5-HT$_{2A}$-selective antagonist MDL100907 demonstrated that the response to 5-HT was elicited through 5-HT$_{2A}$ receptors; N=2 independent experiments (mean +/- SEM).

Next I wanted to determine if activation of a growth factor receptor endogenously expressed in mVSMCs could result in decreased 5-HT$_{2A}$ receptor responsiveness. In this experiment I stimulated endogenous PDGF receptors on mouse smooth muscle cells with two PDGF ligands: human PDGF AB and PDGF BB. These two peptides were chosen because they represent the principal endogenous PDGF ligands and because they are
potent mitogens for murine cells. Unlike the previous experiments, VSMCs were plated into 384-well plates to enable the simultaneous measurement of 5-HT$_{2A}$ receptor activity in RSK2+/+ and RSK2-/- VSMCs in response to both PDGF peptides at multiple time points. These experiments determined that both hPDGF peptides time-dependently decreased 5-HT maximal signaling without significantly affecting potency in RSK2+/+ mVSMCs. Specifically, the largest decreases in maximal efficacy were observed at 1 hr for both peptides ($E_{\text{max}}=99\%$ vs. $84\%$ for untreated and PDGF AB-treated RSK2+/+ MEFs, respectively and $E_{\text{max}}=99\%$ vs. $79\%$ for untreated and PDGF BB-treated RSK2+/+ MEFs, respectively, N=4; $p<0.05$) (Figure 4-7A and C). These data support the studies in MEFs using multiple EGF receptor ligands. In contrast, PDGF pre-treatment in RSK2-/- mVSMCs failed to significantly reduce 5-HT potency and maximal efficacy at all times, including 1hr which was maximal in RSK2+/+ mVSMCs (Figure 4-7B and D).

The data from the RSK2+/+ mVSMCs are consistent with receptor theory since one would predict that a loss of functional receptors in an endogenous system (i.e. low receptor reserve) would result in a significant decrease in the maximal efficacy of a full agonist. This assumption will be further tested below for endogenously expressed P2Y-purinergic receptors. In addition, the data from RSK2-/- mVSMCs support the previous results in RSK2-/- MEFs and confirm that RSK2 is required for RTK-mediated heterologous desensitization of 5-HT$_{2A}$ receptors.
Figure 4.7. RSK2 is required for RTK-mediated heterologous desensitization of 5-HT$_{2A}$ receptors in mouse vascular smooth muscle cells (mVSMCs). RSK2+/+ and RSK2−/− mVSMCs were isolated and treated as described in Chapter 2 MATERIALS AND METHODS. Briefly, serum-starved mVSMCs were pre-treated with 100 ng/mL PDGF AB or PDGF BB peptides for several times and then 5-HT concentration-response curves for Ca$^{2+}$ release were generated. A and C, Pre-treatment of RSK2+/+ mVSMCs with PDGF AB and PDGF BB resulted in time-
dependent downward shifts in 5-HT concentration-response curves. This effect was maximal at 1 hr (shown here) and represented significant decreases in $E_{\text{max}}$ values; N=4 independent experiments (mean +/- SEM), $p<0.05$. B and D, Parallel experiments in RSK2/- mVSMCs did not lead to significant time-dependent shifts in 5-HT concentration-response curves and highlight the requirement for RSK2; N=4 independent experiments (mean +/- SEM), $p<0.05$. These results further confirm the requirement of RSK2 in RTK-mediated heterologous desensitization of 5-HT$_{2A}$ receptors. Furthermore, these results show that PDGF receptors, in addition to EGF receptors, can desensitize endogenous 5-HT$_{2A}$ signaling.

4.8 Pre-Treatment with Insulin does not Lead to Desensitization of 5-HT$_{2A}$ Receptors-Implications for Pathway Specificity

Up to this point I have devised numerous experiments using various RTK ligands (i.e. hEGF, TGF-α, and PDGF), cell lines (MEFs and VSMCs), and GPCR ligands (i.e. 5-HT, 5-methoxyDMT, and lisuride) to directly test the hypothesis that activated RTKs require RSK2 to modulate GPCR signaling. However, it was unknown if insulin receptor activation, which has been shown by others to modulate GPCR signaling, could also modulate 5-HT$_{2A}$ receptor signaling. By performing these experiments I could begin to address questions about the specificity of this novel regulatory pathway. Specifically, is GPCR signaling modulated by the generic activation of RTKs, or do the mechanisms underlying RTK-GPCR cross-talk (i.e. RSK2 activation) engender some level of specificity?
In order to test this, I pre-treated RSK2+/+ and RSK2-/- MEFs with 100 nM insulin for 1 hr. Importantly, I pre-treated RSK2+/+ MEFs with 100 ng/mL hEGF in parallel to ensure that I was observing desensitization. This positive control was crucial to interpreting negative data. As shown in Figure 4-8A and B, insulin pretreatment had no effect on 5-HT concentration-response curves in either RSK2+/+ or RSK2-/- MEFs. Indeed, this result could not be explained by a general deficiency in RTK-mediated heterologous desensitization since hEGF pre-treatment was able to desensitize the 5-HT Ca\(^{2+}\) response (Figure 4-8C). These findings, if true, are very intriguing since they suggest that RTK-mediated heterologous desensitization of GPCRs may be a specific phenomenon. This scenario is likely to be true given the physiological relevance of RTK-GPCR crosstalk as demonstrated for βAR receptor signaling and insulin receptor signaling (108,114). However, several concerns need to be addressed in order to correctly interpret these data. It remains to be determined if insulin treatment is actually activating insulin receptors in MEFs. In addition, it is possible that hEGF activates RSK2 to a greater extent than insulin. Both of these questions will be addressed in future experiments using phospho-specific antibodies that detect activated insulin receptors and activated RSK2.
Figure 4-8. Insulin receptor activation fails to desensitize 5-HT2A receptors.

RSK2+/+ and RSK2-/- mouse embryonic fibroblasts (MEFs) stably expressing FLAG 5-HT2A receptors were treated as detailed in Chapter 2 MATERIALS AND METHODS. Briefly, MEFs were pre-treated with 100 nM insulin for various times and 5-HT-induced intracellular Ca²⁺ release was determined. A and B, 5-HT concentration-response curves for Ca²⁺ release in serum-starved RSK2+/+ MEFs
(A) and RSK2-/- MEFs (B) following 0 min (■), 30 min (○), and 1 hr (▲) insulin pre-treatment show no significant time-dependent attenuation of Ca^{2+} response. Data represent three independent experiments (mean +/- SEM). C, Parallel control experiments in RSK2+/+ MEFs show that the Ca^{2+} response elicited by 10 nM 5-HT was attenuated following 1 hr pre-treatment with 100 ng/mL hEGF. The representative data shown here were replicated in three independent experiments. Together, these results show that insulin receptor activation is unable to desensitize 5-HT_2A receptors, even though the effect of hEGF is intact.

4.9 RSK2 is Required for hEGF-Mediated Heterologous Desensitization of Endogenous P2Y Purinergic Receptor Signaling

In addition to 5-HT_2A receptors, our previous study reported that other GPCRs endogenously expressed in MEFs (e.g. the P2Y purinergic receptor) were negatively regulated by RSK2. Specifically, Sheffler et al. (172) demonstrated that ATP-induced increases in intracellular Ca^{2+} and inositol phosphate (IP) were potentiated in RSK2/-MEFs. Therefore, I wanted to determine if P2Y receptors, like the 5-HT_2A receptors, are desensitized following growth factor receptor activation. I also performed these experiments to determine whether RTK- and RSK2-mediated desensitization of GPCRs is a global phenomenon or specific to certain GPCRs.

In these experiments, I assessed P2Y receptor signaling after 1 hr pre-treatment with 100 ng/mL hEGF. As shown in Figure 4-9A, concentration-response curves for ATP-induced Ca^{2+} release were significantly shifted rightward and downward in the presence of hEGF. This small but significant decrease in potency (7 μM vs. 13 μM for
untreated and hEGF-treated, respectively; N=5, \( p<0.05 \) and decrease in maximal efficacy (\( E_{\text{max}}=100\% \) vs. 82\% in untreated and hEGF-treated, respectively; N=5, \( p<0.05 \)) indicated that the full agonist ATP was desensitized in an experimental system with low (endogenous) receptor expression. These results are in complete agreement with classic receptor theory and support the previous results obtained with partial agonists and mVSMCs (Figure 4-5 and 4-7).

I next wanted to determine if EGF-induced desensitization of purinergic signaling required RSK2. As shown in Figure 4-9B, ATP concentration-response curves were not changed following hEGF pre-treatment. This result suggested that RSK2 was required for RTK-mediated desensitization of P2Y receptors and may, in fact, be a common mechanism whereby RSK2 regulates other GPCRs which show sensitivity to RSK2 expression (e.g. PAR-1 and \( \beta_1 \)AR). This remains to be investigated and should prove to be very interesting for the \( \beta_1 \)AR since this GPCR is already known to be modulated by the growth factor insulin.
Figure 4-9. RSK2 is required for hEGF-mediated heterologous desensitization of P2Y-purinergic receptors. RSK2+/+ and RSK2−/− mouse embryonic fibroblasts (MEFs) stably expressing 5-HT2A receptors were treated as detailed in Chapter 2 MATERIALS AND METHODS. Briefly, MEFs were pre-treated with 100 ng/mL hEGF for various times and ATP-induced intracellular Ca$^{2+}$ release was determined. A, The concentration-response curve for ATP-mediated Ca$^{2+}$ release in serum-starved RSK2+/+ MEFs is shifted rightward and downward following 1 hr (o) hEGF pre-treatment. These shifts denote significant decreases in 5-HT potency and maximal efficacy; N=5 independent experiments (mean +/- SEM), p<0.05. B, The concentration-response curve for ATP-mediated Ca$^{2+}$ release in serum-starved RSK2−/− MEFs is not significantly shifted following 1 hr (o) hEGF pre-treatment. These results show that RSK2 is required for RTK-mediated heterologous
desensitization of P2Y-purinergic receptors and may represent a common mechanism whereby RTKs modulate GPCR signaling.

4.10 Discussion

In this chapter I present multiple lines of evidence to support the hypothesis that RTKs act via RSK2 to modulate GPCR signaling. This novel mechanism was the result of recent data showing that EGF receptor activation phosphorylated the 5-HT$_{2A}$ receptor at Ser 314-a site that is known to modulate 5-HT$_{2A}$ receptor signaling in MEFs (290). Taking this into consideration, it seemed entirely plausible that RTK activation, acting via RSK2, could phosphorylate 5-HT$_{2A}$ receptors and facilitate receptor desensitization. Moreover, this mechanism was consistent with recent reports of a novel regulatory paradigm whereby RTK activation modulates GPCR signaling. Specifically, RTK activation was shown to profoundly affect the signaling of the β$_1$, β$_2$, and α$_{1B}$-adrenergic receptors, as well as 5-HT$_{2C}$ receptors. Thus, given the important physiological implications behind RTK regulation of GPCR signaling as demonstrated for insulin receptor-mediated inhibition of β$_2$AR signaling (114), it seemed likely that additional GPCRs could be regulated by RTKs.

To expand, I first demonstrated that hEGF- and TGF-α-mediated EGF receptor activation resulted in a time- and concentration-dependent desensitization of 5-HT$_{2A}$ receptors in RSK2+/+ MEFs. For the full agonist 5-HT, this desensitization manifested as significant rightward shifts in concentration-response curves without changes in maximal efficacy. These dextral shifts signified decreases in potency which were the result of a loss of functional receptors (320). Despite shifts in potency, the full agonist 5-HT, by
virtue of its large receptor reserve in MEF over-expressing 5-HT$_{2A}$ receptors, signaled maximally irrespective of changes in functional receptors. On the other hand, partial agonists have a lower receptor reserve and are more sensitive to changes in the number of functional receptors. Therefore concentration-response curves for partial agonists are more likely to exhibit shifts in both potency and efficacy. I applied these principles in subsequent experiments to verify that 5-HT$_{2A}$ receptors were desensitized following RTK activation. In these studies I observed significant decreases in the maximal efficacies of the partial agonists 5-methoxyDMT and lisuride following hEGF pre-treatment. These results, and those obtained with the full agonist 5-HT, are consistent with a loss of functional 5-HT$_{2A}$ receptors and can be attributed to receptor desensitization given the extremely rapid timescale of the Ca$^{2+}$ measurements (320).

Parallel experiments in RSK2/- MEFs indicated that RTK-mediated desensitization of 5-HT$_{2A}$ receptors requires RSK2. In these experiments EGF receptor activation did not significantly shift 5-HT concentration-response curves. In fact, 5-HT concentration-response curves generated after hEGF or TGF-α pre-treatment were indistinguishable from curves generated from untreated RSK2/- MEFs. I attributed these differences to the absence of RSK2 since previous microarray data did not detect any differences in EGF receptor expression (Dr. Douglas J. Sheffler, unpublished observations). However, since we have not biochemically evaluated EGF receptor expression, further studies are needed to evaluate this hypothesis.

In this chapter I also demonstrate that endogenous 5-HT$_{2A}$ receptor signaling is modulated by RTKs and that this regulation requires RSK2. Using VSMCs isolated from RSK2 +/+ and RSK2/- mice, I determined that endogenous 5-HT$_{2A}$ receptors could be
desensitized following PDGF receptor activation. Similar to the behavior exhibited by the partial agonists 5-methoxyDMT and lisuride in RSK2+/+ MEFs, PDGF receptor activation led to significant decreases in 5-HT maximal efficacy. According to receptor theory, this suggested that the small endogenous pool of 5-HT$_{2A}$ receptors was desensitizing following RTK activation. As determined previously, significant decreases in maximal efficacy were not observed in RSK2-/− mVSMCs which further establish a role for RSK2 in RTK-GPCR cross-talk.

Interestingly, and in stark contrast to experiments targeting EGF receptors and PDGF receptors, the data presented in Figure 4-9 show that insulin failed to significantly shift 5-HT concentration-response curves in both RSK2+/+ and RSK2-/− MEFs. These negative results are verified by the ability for hEGF pre-treatment to decrease 5-HT signaling in parallel experiments. Thus, at first glance it seems that insulin receptor activation cannot desensitize 5-HT$_{2A}$ receptors in MEFs. The reason for this remains unknown, although it is possible that insulin receptors do not activate RSK2 to the same extent as EGF receptors and PDGF receptors in MEFs. This is supported by evidence showing that insulin does not robustly activate the ERK/MAPK cascade in some cell types (260,321).

In a final set of experiments, I determined that RSK2 was required for RTK-mediated heterologous desensitization of P2Y-purinergic receptors. P2Y receptors are endogenously expressed in MEFs, and in accordance with receptor theory, hEGF pre-treatment significantly decreased both ATP potency and maximal efficacy in RSK2+/+ MEFs. This receptor behavior agrees completely with the predictions of receptor theory and is consistent with loss of functional receptors in an endogenous system with little or
no receptor reserve. Considering that our laboratory has previously identified these receptors as being regulated by RSK2 (172), it is possible that other GPCRs which are regulated by RSK2 (i.e. β₁AR and PAR-1 thrombin receptor) can be desensitized via RTK activation. In the case of β₁ARs it would be interesting to determine if RSK2 serves as an additional regulatory intermediate, since it is already known that the insulin-like growth factor-1 receptor regulates β₁AR signaling through activation of PI3K and Akt (108).

It is known that 5-HT₂A receptors transactivate the MAPK/ERK cascade. Therefore, the results presented here are consistent with the intriguing possibility that RSK2 is activated downstream of ERK, thereby exerting negative feedback control on 5-HT₂A signaling (i.e. homologous desensitization, Figure 4-10). However, metabolic labeling experiments do not detect 5-HT₂A phosphorylation under basal conditions or following stimulation with 5-HT (Dr. Bryan L. Roth, unpublished observations) (190). It remains a possibility that the methods employed here are not sensitive enough to detect low levels of receptor phosphorylation; therefore our laboratory will actively pursue additional studies to determine if RSK2 plays an important role in mediating homologous desensitization of the 5-HT₂A receptor.

On the other hand, our data are consistent with a heterologous desensitization model in which growth factors activate RTKs and downstream MAPKs, which in turn activate RSK2 and phosphorylate the 5-HT₂A receptor to modulate its function (Figure 4-10). It is well-established that RTK activation modulates β₁AR, β₂AR, and α₁b-adrenergic receptor signaling(108-112), as well as 5-HT2C receptor signaling (113). Notably, it is accepted that this cross-talk between the insulin receptor and adrenergic receptors serve
as a mechanism for functional antagonism of glucose homeostasis (114). It is attractive to speculate that growth factor signaling may be relevant for regulating the 5-HT$_2$A receptor \textit{in vivo} and is consistent with the role of RTK signaling in maintaining normal brain function (322,323). Significantly, the model presented in this chapter may have far-reaching implications for human diseases associated with 5-HT$_2$A dysregulation since it has been recently shown that some RTKs are considered susceptibility factors for schizophrenia (323,324). Indeed, the data presented in this chapter and previous chapters are extremely exciting and have opened up new avenues of discovery for 5-HT$_2$A research. These future studies will be explored in detail in \textbf{Chapter 6}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4-10.png}
\caption{\textbf{Figure 4-10. Model describing RTK-mediated modulation of 5-HT$_2$A receptor signaling.} GPCR-receptor tyrosine kinase (RTK) crosstalk can occur in two directions. First, in addition to 5-HT$_2$A receptor signaling through G proteins (center), 5-HT$_2$A receptors are known to activate the ERK/MAPK cascade and explains many effects of 5-HT including mitogenesis and early response gene}
\end{figure}
expression. Several mechanisms account for this including ADAM17 metalloprotease-mediated transactivation of the epidermal growth factor (EGF) receptor, generation of reactive oxygen species (ROS), and activation of NHE1 and MEK1 (left). Secondly, RTK activation is known to regulate GPCR signaling (i.e. β- and α-adrenergic receptors). In this thesis I show that activation of the EGF receptor leads to RSK2 activation and subsequent phosphorylation of Ser314 within the 5-HT2A receptor i3 loop-a site shown to regulate 5-HT2A signaling (Chapters 3 and 4). Therefore it was hypothesized that RTKs act via RSK2 to modulate GPCR signaling. Certainly, the data presented in this chapter support the requirement for RSK2 in RTK-mediated heterologous desensitization of 5-HT2A receptors (right). Importantly, this novel regulatory pathway provides an explanation for RTK-mediated desensitization of P2Y-purinergic receptors and suggests a common mechanism whereby RSK2 modulates GPCR responsiveness.
CHAPTER 5: RSK2-DEPENDENT ALTERATIONS IN THE RELATIVE RANK ORDER EFFICACY OF 5-HT$_{2A}$ SEROTONIN RECEPTOR AGONISTS

5.1 Introduction and Rationale

As mentioned briefly in Chapter 1, the concept of functional selectivity has now thoroughly supplanted the previously entrenched notion of intrinsic efficacy by explaining how agonists and antagonists can exhibit a range of efficacies for distinct receptor-mediated responses. It was originally thought that a ligand’s ability to impart (or reduce) a stimulus once bound to the receptor (i.e. intrinsic efficacy, (5)) is an inherent property of the ligand-receptor complex and is system-independent (i.e. relative order of efficacy is static across all receptor responses) (325). However, a plethora of recent studies on receptor tyrosine kinases and GPCRs including neurotensin, dopamine, opioid, cannabinoid, chemokine, beta-adrenergic, vasopressin, somatostatin, and serotonin receptors have shown that ligands exhibit a wide range of efficacies for different receptor behaviors (i.e. relative order of efficacy is dynamic across various receptor responses) (6,57,167,326-331). To address these observations and to provide a unifying conceptual framework, the related concepts of ‘functional selectivity’ (332), ‘agonist-directed trafficking of receptor stimulus’ (333,334), ‘biased agonism’ (335), and ‘pluridimensionality of signaling’ (336) (collectively referred to here as ‘functional selectivity’) have emerged.

The capacity for ligands to elicit a gradient of receptor behaviors is well-documented for the G$_{q/11}$-coupled 5-HT$_{2A}$ and 5-HT$_{2C}$ serotonin receptors. These receptors
are essential for mediating various functions of 5-HT in both central and peripheral tissues (e.g. modulation of mood and perception, regulation of appetite, and platelet aggregation) and are targeted by multiple drugs (135,337). In what are now considered classic studies, the laboratory of Clarke and Berg (167,331) convincingly demonstrated that the relative rank orders of efficacy for chemically diverse agonists at 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors were reversed between PLCβ-mediated inositol phosphate (IP) accumulation and PLA2-mediated arachidonic acid (AA) release. In a similar way, Kurrasch-Orbaugh et al. (330) reported that rank orders of efficacy were reversed for several classes of 5-HT$_{2A}$ agonists when comparing IP accumulation and AA release. Significantly, the pleiotropic nature of 5-HT$_2$ ligands was highlighted in a recent study wherein the 5-HT$_{2C}$ selective ‘antagonist’ SB242084 both antagonizes 5-HT$_{2C}$-mediated AA release and promotes IP accumulation (338). Additionally, some 5-HT$_{2A}$-selective ligands behave as ‘inverse agonists’ at IP accumulation and ‘agonists’ at receptor internalization in vitro and in vivo (145,198). Clearly these pathway-specific reversals in relative efficacy are incompatible with classical notions of intrinsic efficacy and, in fact, are considered benchmark examples of functional selectivity.

As seen for 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, bona fide receptor-based functional selectivity manifests as a reversal in relative efficacies at different pathways. This behavior is not predicted by classic intrinsic efficacy and can only be explained by agonists stabilizing or promoting different receptor active states (335). It follows, then, that the functional selectivity concept, unlike the concept of ligand intrinsic efficacy, ascribes quality to efficacy. Thus, ligand-specific receptor conformations can elicit a plethora of effector readouts including G protein activation, phosphorylation,
desensitization and internalization, formation of receptor dimers and oligomers, and interaction with auxiliary membrane and cytosolic proteins (325). Therefore, ligand efficacy is conditional upon the presence (or absence) of auxiliary modifying proteins within the cellular milieu (otherwise known as ‘conditional efficacy’). Indeed, conditional efficacy has been demonstrated for arrestin-dependent events in vitro and in vivo (339,340). Thus, it is likely that many intracellular proteins including arrestins, kinases and scaffolding proteins can differentially modulate ligand efficacy (215,341).

As detailed previously in Chapter 1, the ERK/MAPK effector, RSK2 was identified as a novel 5-HT2A interacting partner and regulator of GPCR signaling. Specifically, 5-HT2A-mediated maximal increases in IP accumulation and intracellular Ca2+ release were significantly potentiated in 5-HT-treated RSK2-/− mouse embryonic fibroblasts (MEFs) compared to RSK2+/+ MEFs (172). In Chapter 3, I showed via multiple lines of evidence that RSK2 kinase activity was required for receptor regulation and that RSK2 directly phosphorylates the 5-HT2A receptor third intracellular (i3) loop at Ser314 to modulate the signaling of several known 5-HT2A agonists (290). Based on these findings and the mounting evidence for conditional efficacy I hypothesized that 5-HT2A agonist signaling would be disproportionately affected by changes in RSK2 expression. To test this hypothesis I initiated a focused screen evaluating the effect of RSK2 expression (i.e. in RSK2+/+ and RSK2-/− MEFs) on the signaling of a chemically diverse panel of 5-HT2A receptor agonists at several readouts of 5-HT2A signaling (e.g. IP accumulation, Ca2+ release, and ERK1/2 phosphorylation). Importantly, this screen incorporated the use of established and novel high-throughput technologies thus enabling
the generation of full concentration-response curves for accurate potency (EC$_{50}$) and maximal efficacy (E$_{max}$) determinations.

5.2 Validation of a Novel High Content Microscopic Approach for ERK1/2 Phosphorylation.

For these studies, I employed high-throughput technologies to rapidly measure 5-HT$_{2A}$-mediated IP accumulation and Ca$^{2+}$ release, and also developed a novel triple-label high content microscopic approach to measure 5-HT$_{2A}$-mediated ERK1/2 phosphorylation in RSK2+/+ and RSK2-/- MEFs. First, this technique which is both powerful and amenable to high-throughput analysis, is a vast improvement over the existing low-throughput Western blotting technique. Furthermore, complementary approaches of high content microscopy and image analysis software allow the researcher to extract a variety of information from immunofluorescence images such as subcellular localization of the signaling event (e.g. translocation of ERK1/2 to the nucleus). In the approach outlined here (Figure 5-1A), fixed cells were triple-labeled with nuclear (Hoechst) and membrane (Concanavalin A) dyes thereby allowing us to measure average fluorescence intensity of the signal channel (594 nm) within nuclear, cytoplasmic, and whole cell masks.

This technique was used to generate concentration-response curves for ERK1/2 phosphorylation in RSK2+/+ and RSK2-/- MEFs following 5 min agonist treatment (Figure 5-1B), which was validated to be maximal in both Western blot and high content microscopic assays (Figure 5-2). In addition, control experiments ensured that ERK1/2
phosphorylation was mediated through 5-HT$_{2A}$ receptors by blocking agonist responses with the 5-HT$_{2A}$ selective antagonist MDL100907 (shown for 5-HT, inset Figure 5-1B).

Figure 5-1. Development of a novel triple-label high content microscopic assay in 384-well plates (A) to rapidly measure ERK1/2 phosphorylation in RSK2+/+ and RSK2-/- MEFs (B). A, Diagram showing stepwise generation of nuclear (Hoechst-380 nm), whole cell (Concanavalin A-488 nm), and cytoplasmic cell masks subsequently used to measure fluorescence intensity of the signal channel (594 nm) in distinct cellular regions. This approach incorporates the nearly limitless...
application of state-specific antibodies (e.g. phospho-specific) and fluorescently labeled proteins to measure various receptor responses. Moreover, segmenting the cell into distinct regions provides us with the opportunity to extract a variety of signaling phenotypes with customizable CellProfiler image analysis software. B, I used this technology to generate full concentration response curves for ERK1/2 phosphorylation in RSK2+/+ and RSK2-/− MEFs after agonist treatment. The workflow shown here represents the steps used to measure ERK1/2 phosphorylation (594 nm, see representative images) within whole cell masks after 5-HT treatment. Nonlinear regression curve fits (bottom) show the quantified data (mean +/- SEM) for 5-HT-induced ERK1/2 phosphorylation in RSK2+/+ (■) and RSK2-/− (○) MEFs. Also shown are representative results (bar graph) wherein the 5-HT2A selective antagonist MDL100907 (1 µM) blocked the response to 5-HT (EC80 concentration). Identical results were obtained for all agonists at measures of IP accumulation, Ca2+ release, and ERK1/2 phosphorylation.
Figure 5-2. The time course of 5-HT-induced ERK1/2 phosphorylation in RSK2+/+ MEFs is similar between Western blot and triple-label high content microscopic techniques. As shown for both techniques (Western blot (■) and high content microscopy (○)), maximal 5-HT-induced (10μM) ERK1/2 phosphorylation occurred within 5 min for RSK+/+ MEFs. These results represent the mean +/- SEM of at least two independent experiments. I also determined via high content microscopy that ERK1/2 phosphorylation was maximal within 5 min in RSK2+/+ and RSK2-/- MEFs for the agonists (10μM) 5-HT, DOI, quipazine, 5-methoxyDMT, lisuride, m-CPP, SCH-23390, α-methyl5-HT, and MK212 (data not shown).

5.3 5-HT$_{2A}$ Agonist Responses to a Variety of Agonists are Augmented by Genetic Deletion of RSK2.

The first step in this experiment was to determine if the previously reported potentiation of agonist signaling in the absence of RSK2 could be seen with a structurally diverse family of 5-HT$_{2A}$ agonists. I discovered, in confirmation of our previous studies
that genetic deletion of RSK2 significantly increased the relative efficacies of structurally diverse 5-HT2A agonists, with few exceptions. Moreover, this potentiation was not due to differences in receptor expression since EEDQ receptor normalization experiments showed that 5-HT2A signaling was potentiated in RSK2-/- MEFs relative to RSK2+/+ MEFs under conditions of equal receptor expression (Figure 5-3). Specifically, the reference full agonist 5-HT, along with α-methyl-5-HT and DOI, elicited significantly greater maximal increases in IP accumulation, Ca2+ release, and ERK1/2 phosphorylation in RSK2-/- MEFs relative to RSK2+/+ MEFs (Tables 5-1, 5-2, and 5-3). Additionally, the relative efficacies of quipazine, 5-methoxy-DMT, lisuride, and m-CPP were significantly increased at all three readouts in RSK2-/- MEFs (Tables 5-1, 5-2, and 5-3).

In contrast, the partial agonists SCH-23390 and MK212 showed significant potentiation for IP accumulation and ERK1/2 phosphorylation, but not Ca2+ release.

The relative agonist potencies were not, however, significantly different between RSK2+/+ and RSK2-/- MEFs at any readout, with a few exceptions. The exceptions included the full agonists 5-HT and α-methyl5-HT which were significantly more potent for IP accumulation in RSK2-/- MEFs, and the partial agonist quipazine which was significantly more potent for Ca2+ release in RSK2+/+ MEFs (Tables 5-1, 5-2, and 5-3). Taken together, these data showed that RSK2 expression consistently modulated agonist efficacy, whereas agonist potency remained largely unaffected.
Figure 5-3. 5-HT$_{2A}$ receptor signaling is potentiated in RSK2/-/ MEFs relative to RSK2+/+ MEFs after normalization of receptor expression. RSK2+/+ and RSK2-/- MEFs stably expressing FLAG 5-HT$_{2A}$ receptors were treated with various concentrations of the irreversible alkylating agent EEDQ in order to assess 5-HT$_{2A}$ Ca$^{2+}$ signaling (A) under conditions of equal receptor expression (B). A, These studies were carried out as described in Chapter 2. Briefly, RSK2+/+ and RSK2-/- MEFs were plated for FLIPR Ca$^{2+}$ mobilization and radioligand binding experiments and then treated in parallel with EEDQ (1 µM to 100 µM). Concentration-response curves generated from wells treated with concentrations of EEDQ that produced equal levels of receptor expression were compared between cell lines. Under these conditions of equal receptor expression it was possible to directly assess the contribution of RSK2 to 5-HT$_{2A}$ signaling. Normalized values were expressed as percent of the maximal signaling of 5-HT in untreated RSK2+/+ MEFs. B, As shown here, EEDQ decreased 5-HT$_{2A}$ receptor numbers in a concentration-dependent manner, and were considered to be equal at 10 µM for
RSK2\/+ MEFs and 10 µM for RSK2\/- MEFs. Specific binding values were normalized to membrane protein present in each well.

Table 5-1

Relative agonist potency and efficacy values for 5-HT\(_{2A}\)-mediated IP accumulation.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Relative agonist potency(^a), (EC_{50}) nM (pEC(_{50}) ± SEM)</th>
<th>Relative agonist efficacy(^a), (E_{\text{max}}) ± SEM</th>
<th>F-test, p value</th>
<th>F-test, p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>RSK2/+ MEFs: 134 (-6.87 +/- 0.03) RSK2/- MEFs: 57.8 (-7.24 +/- 0.11) 0.0309</td>
<td>RSK2/+ MEFs: 99.1 +/- 1.2 RSK2/- MEFs: 209 +/- 8.5 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DOI</td>
<td>12.4 (-7.91 +/- 0.10) 8.51 (-8.07 +/- 0.10) 0.7145</td>
<td>71.7 +/- 2.4 211 +/- 13 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Quipazine</td>
<td>188 (-6.73 +/- 0.09) 140 (-6.85 +/- 0.15) 0.7010</td>
<td>82.7 +/- 2.9 220 +/- 14 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5-methoxyDMT</td>
<td>590 (-6.23 +/- 0.13) 386 (-6.413 +/- 0.15) 0.6487</td>
<td>66.6 +/- 4.0 224 +/- 14 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lisuride</td>
<td>3.52 (-6.45 +/- 0.57) 5.99 (-6.22 +/- 0.20) 0.7450</td>
<td>17.1 +/- 2.6 63.1 +/- 3.8 &lt;0.000</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>m-CPP</td>
<td>167 (-6.78 +/- 0.19) 231 (-6.64 +/- 0.09) 0.5364</td>
<td>27.8 +/- 2.4 90.0 +/- 3.6 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SCH23390</td>
<td>16.3 (-7.79 +/- 0.30) 24.6 (-7.61 +/- 0.08) 0.5342</td>
<td>14.0 +/- 1.5 33.9 +/- 1.1 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>α-methyl5-HT</td>
<td>178 (-6.75 +/- 0.04) 68.9 (-7.16 +/- 0.05) &lt;0.0001</td>
<td>95.4 +/- 1.9 134 +/- 3.0 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MK212</td>
<td>3990 (-5.47 +/- 0.09) 2930 (-5.63 +/- 0.05) 0.1148</td>
<td>80.4 +/- 5.7 152 +/- 5.0 &lt;0.000</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Relative agonist potencies (EC\(_{50}\)) and efficacies (\(E_{\text{max}}\)) represent the average of four to five independent experiments.

\(^b\)pEC\(_{50}\) values are represented as –log of EC\(_{50}\) in M
Table 5-2
Relative agonist potency and efficacy values for 5-HT\textsubscript{2A}-mediated intracellular Ca\textsuperscript{2+} release.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Relative agonist potency\textsuperscript{a}, EC\textsubscript{50} nM (pEC\textsubscript{50} ± SEM\textsuperscript{b})</th>
<th>Relative agonist efficacy\textsuperscript{a}, E\textsubscript{max} +/- SEM</th>
<th>F-test, p value</th>
<th>F-test, p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>7.79 (-8.11 +/- 0.07) 10.2 (-7.99 +/- 0.12)</td>
<td>97.0 +/- 2.2 202 +/- 12.5</td>
<td>0.6969</td>
<td>&lt;0.0001 1.5</td>
</tr>
<tr>
<td>DOI</td>
<td>2.81 (-8.55 +/- 0.12) 4.68 (-8.33 +/- 0.11)</td>
<td>87.3 +/- 2.9 141 +/- 4.6</td>
<td>0.2309</td>
<td>&lt;0.0001 1.6</td>
</tr>
<tr>
<td>Quipazine</td>
<td>22.8 (-7.64 +/- 0.12) 62.3 (-7.21 +/- 0.09)</td>
<td>88.1 +/- 3.6 157 +/- 5.7</td>
<td>0.0114</td>
<td>&lt;0.0001 1.8</td>
</tr>
<tr>
<td>5-methoxy DMT</td>
<td>60.0 (-7.22 +/- 0.08) 58.2 (-7.24 +/- 0.14)</td>
<td>83.2 +/- 2.6 145 +/- 7.8</td>
<td>0.9509</td>
<td>&lt;0.0001 1.7</td>
</tr>
<tr>
<td>Lisuride</td>
<td>985 (-6.01 +/- 0.17) 1023 (-5.99 +/- 0.12)</td>
<td>29.1 +/- 2.7 58.7 +/- 3.8</td>
<td>0.9405</td>
<td>&lt;0.0001 2.0</td>
</tr>
<tr>
<td>m-CPP</td>
<td>155 (-6.81 +/- 0.18) 234 (-6.63 +/- 0.31)</td>
<td>60.5 +/- 5.0 116 +/- 17</td>
<td>0.6959</td>
<td>0.0039 1.9</td>
</tr>
<tr>
<td>SCH-23390</td>
<td>329 (-6.48 +/- 0.32) 197 (-6.71 +/- 0.34)</td>
<td>25.7 +/- 4.0 33.2 +/- 5.2</td>
<td>0.6864</td>
<td>0.2974 1.3</td>
</tr>
<tr>
<td>α-methyl 5-HT</td>
<td>25.0 (-7.60 +/- 0.12) 21.9 (-7.66 +/- 0.41)</td>
<td>106 +/- 4.5 212 +/- 32</td>
<td>0.9401</td>
<td>0.0046 2.0</td>
</tr>
<tr>
<td>MK212</td>
<td>817 (-6.09 +/- 0.18) 846 (-6.07 +/- 0.33)</td>
<td>72.5 +/- 7.8 168 +/- 33</td>
<td>0.9793</td>
<td>0.1035 2.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Relative agonist potencies (EC\textsubscript{50}) and efficacies (E\textsubscript{max}) represent the average of four to six independent experiments.

\textsuperscript{b}pEC\textsubscript{50} values are represented as –log of EC\textsubscript{50} in M.
Table 5-3

Relative agonist potency and efficacy values for 5-HT$_{2A}$-mediated ERK1/2 phosphorylation.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Relative agonist potency$^a$, EC$<em>{50}$ nM (pEC$</em>{50}$ ± SEM)$^b$</th>
<th>Relative agonist efficacy$^a$, $E_{max}$ +/-SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RSK2+/+ MEFs</td>
<td>RSK2/- MEFs</td>
</tr>
<tr>
<td>5-HT</td>
<td>6.51 (-8.19 +/- 0.08)</td>
<td>3.92 (-8.41 +/- 0.11)</td>
</tr>
<tr>
<td>DOI</td>
<td>0.370 (-9.43 +/- 0.16)</td>
<td>0.889 (-9.05 +/- 0.22)</td>
</tr>
<tr>
<td>Quipazine</td>
<td>2.42 (-8.62 +/- 0.17)</td>
<td>4.24 (-8.37 +/- 0.20)</td>
</tr>
<tr>
<td>5-methoxy DM</td>
<td>8.23 (-8.08 +/- 0.19)</td>
<td>7.45 (-8.13 +/- 0.19)</td>
</tr>
<tr>
<td>Lisuride</td>
<td>7.39 (-8.13 +/- 0.21)</td>
<td>2.27 (-8.64 +/- 0.19)</td>
</tr>
<tr>
<td>m-CPP</td>
<td>21.6 (-7.67 +/- 0.18)</td>
<td>21.6 (-7.67 +/- 0.19)</td>
</tr>
<tr>
<td>SCH-23390</td>
<td>21.2 (-7.67 +/- 0.29)</td>
<td>3.65 (-8.44 +/- 0.23)</td>
</tr>
<tr>
<td>α-methyl 5-HT</td>
<td>3.07 (-8.51 +/- 0.19)</td>
<td>3.91 (-8.41 +/- 0.17)</td>
</tr>
<tr>
<td>MK212</td>
<td>133 (-6.88 +/- 0.13)</td>
<td>42.5 (-7.37 +/- 0.14)</td>
</tr>
</tbody>
</table>

$^a$Relative agonist potencies (EC$_{50}$) and efficacies (E$_{max}$) represent the average of four to six independent experiments.

$^b$pEC$_{50}$ values are represented as –log of EC$_{50}$ in M.
Indeed, plots of relative efficacy vs. potency for IP accumulation and Ca\(^{2+}\) release illustrated that RSK2 deletion significantly potentiated agonist efficacies, with few changes in agonist potency (Figure 5-4 A-B). As shown in Figure 5-4 C, RSK2 deletion globally potentiated agonist-mediated ERK1/2 phosphorylation with little distinction between full and partial agonists. In fact, global potentiation of ERK1/2 phosphorylation in RSK2 -/- MEFs is consistent with removal of feedback inhibition on the ERK/MAPK pathway since it is known that RSK2 phosphorylates Sos, thereby decreasing Ras activation (232).

**Figure 5-4.** Plots of relative efficacy (\(E_{\text{max}}, 5\text{-HT set to } 100\%\)) vs. potency (\(pEC_{50}\)) for IP accumulation (A), Ca\(^{2+}\) release (B), and ERK1/2 phosphorylation (C) in RSK2+/+(WT, ■) and RSK2-/- (KO, ○) MEFs. \(E_{\text{max}}\) and \(pEC_{50}\) values were calculated via nonlinear regression as reported in Tables 5-1, 5-2 and 5-3. Values represent the mean +/- SEM of three to six independent experiments performed in duplicate. Agonists tested were: 5-HT, DOI, quipazine, 5-methoxyDMT, lisuride, m-CPP, SCH-23390, \(\alpha\)-methyl5-HT, and MK212.
I also generated $E_{\text{max}}^{\text{RSK2-/-}}/E_{\text{max}}^{\text{RSK2+/+}}$ values at each receptor response in order to measure an agonist’s propensity to signal in the absence of RSK2. Interestingly, I found that $E_{\text{max}}^{\text{RSK2-/-}}/E_{\text{max}}^{\text{RSK2+/+}}$ values differed for each agonist and response (Tables 5-1, 5-2, and 5-3), with the largest changes observed for IP accumulation. Thus it appeared that each agonist exhibited a characteristic sensitivity to RSK2 regulation. Specifically, the partial agonists 5-methoxy-DMT (3.4-fold), m-CPP (3.2-fold), and lisuride (3.7-fold) differed substantially from the reference full agonist 5-HT (2.1-fold) in their abilities to accumulate IP (Figure 5A and D). In fact, m-CPP and lisuride, whose relative efficacies were extremely low in RSK2+/+ MEFs, behaved as moderate to full agonists in RSK2-/- MEFs (i.e. compared to the reference agonist 5-HT in RSK2+/+ MEFs).

These data could be explained by the possibility that partial agonists, as a class, signaled better in RSK2 -/- MEFs. This is not unfounded since differences in stimulus-response coupling, often encountered when comparing different cell lines or tissues, manifest as large increases in partial agonist efficacy. However, as shown in Figure 5E, the relative efficacy of the weak partial agonist SCH-23390, which was comparable to lisuride in RSK2+/+ MEFs, increased only 2.4-fold in RSK2 -/- MEFs. In addition, the relative efficacy of MK212 was weakly potentiated (1.9-fold; not shown), suggesting that increased receptor responsiveness was not exclusive to partial agonists and rules out the possibility of any intrinsic differences in stimulus-response coupling when comparing RSK2+/+ and RSK2 -/- MEFs.

In contrast to partial agonists, highly efficacious ligands such as 5-HT (2.1-fold) and α-methyl5-HT (1.4-fold) were not differentially sensitive to RSK2 deletion. In fact, α-methyl5-HT was the least responsive for IP accumulation in RSK2 -/- MEFs (Figure 5-
Importantly, this effect did not result from saturation of the response system since 5-HT was significantly more efficacious than α-methyl5-HT in RSK2 -/- MEFs (relative $E_{\text{max}} = 134 +/- 3.0\%$ vs. $209 +/- 8.5\%$ for α-methyl5-HT and 5-HT, respectively; $p<0.05$). Thus, surprisingly, α-methyl5-HT did not behave as a full agonist in the RSK2/- MEFs. These data support the notion that some agonists are differentially responsive to RSK2 deletion, and in the case of IP accumulation, accounts for reversals in relative rank order of efficacy between RSK2+/+ and RSK2 -/- MEFs (i.e. functional selectivity, see below).

**Figure 5-5.** Differences in $E_{\text{max}} \text{RSK2}^{-/-}/E_{\text{max}} \text{RSK2}^{+/+}$ values suggest that 5-HT$_{2A}$ agonists are differentially responsive to RSK2 regulation. Shown here are concentration-response curves for IP accumulation in RSK2+/+ (WT, ■) and RSK2/- (KO, ○) MEFs in following 60 min treatment with 5-HT (A), 5-methoxyDMT (B), m-CPP (C), lisuride (D), SCH-23390 (E), and α-methyl5-HT (F). Relative efficacy values ($E_{\text{max}}$, 5-HT set to 100%) were determined via nonlinear regression and were significantly potentiated for all agonists in RSK2/- MEFs, as shown in Table 5-1.
Values represent the mean +/- SEM of four to five independent experiments performed in duplicate. \( E_{\text{max}}^{\text{RSK2-/-}} / E_{\text{max}}^{\text{RSK2+/+}} \) values were calculated for each agonist and are shown next to each plot as a measure of an agonist’s propensity to signal in the absence of RSK2.

5.4 5-HT\textsubscript{2A} Agonists are Functionally Selective for ERK1/2 Phosphorylation in RSK2+/+ MEFs.

My main goal was to test the hypothesis that 5-HT\textsubscript{2A} agonist signaling is differentially modulated by RSK2. Therefore I needed to determine if the relative rank orders of efficacy change when comparing 5-HT\textsubscript{2A} signaling readouts in RSK2+/+ and RSK2-/- MEFs. Importantly, I chose to focus on agonist efficacy and not potency since measurements of relative efficacy solely reflect the intrinsic efficacy of the agonist while measures of potency are influenced by both agonist affinity and efficacy. Put simply, relative efficacy values are derived from saturating concentrations of agonist and can therefore be estimated independently of changes in agonist affinity between cell lines.

In order to test this hypothesis I generated statistically rigorous rank orders of efficacy for each receptor response in RSK2+/+ and RSK2 -/- MEFs to identify changes in rank order of efficacy 1) between all receptor responses in RSK2+/+ MEFs and 2) between RSK2+/+ and RSK2 -/- MEFs at each receptor response. In this approach I assigned agonists to statistically homogeneous groups via the Tukey-Kramer method (as described in Chapter 2) such that significant differences in rank order (set as \( p<0.05 \)) are denoted by changes in group membership (Table 5-4). This analysis is a significant improvement to the standard assignment of rank order in that it applies an unbiased
statistical criterion, in this case, an unplanned multiple comparisons test for unequal sample sizes.

A comparison of agonist responses in RSK2+/+ MEFs revealed that the relative efficacies did not differ substantially between the dependent measures of IP accumulation and Ca	extsuperscript{2+} release, in agreement with other studies (167) (Table 5-4). By contrast, the relative efficacies of some agonists differed significantly when comparing IP accumulation and ERK1/2 phosphorylation (Table 5-4), as illustrated in Figure 5-6. Presented in this way, it is clear that lisuride and m-CPP were much more efficacious for ERK 1/2 phosphorylation than IP accumulation. Specifically, lisuride and m-CPP were weak to moderate partial agonists for IP accumulation and Ca	extsuperscript{2+} release, but maximally activated ERK1/2 in RSK2+/+ MEFs (Figure 5-7A and B). In agreement with several other studies (167,330), lisuride only weakly activated 5-HT	extsubscript{2A}-mediated IP accumulation (Relative E	extsubscript{max}=17.1 +/- 2.6%, N=4) and Ca	extsuperscript{2+} release (Relative E	extsubscript{max}= 29.1 +/- 2.7%, N=4). However, at ERK1/2 phosphorylation lisuride signaled similarly to the reference full agonist 5-HT (relative E	extsubscript{max}=90.9 +/- 8.3% and 99.1 +/- 3.5% for lisuride and 5-HT, respectively; p>0.05). Similar observations were made for the partial agonist m-CPP, in which m-CPP was a partial agonist for IP accumulation (relative E	extsubscript{max}=27.8 +/- 2.4%, N=5) and Ca	extsuperscript{2+} release (relative E	extsubscript{max}=60.5 +/- 5.0%); by contrast, m-CPP was equal to 5-HT for inducing ERK phosphorylation (relative E	extsubscript{max}=105 +/- 9.0% and 99.1 +/- 3.5% for m-CPP and 5-HT, respectively; p>0.05).

The observation that the relative efficacies of partial agonists were not uniformly increased for ERK1/2 phosphorylation in RSK2+/+ MEFs suggested that the ERK1/2 pathway was not more efficiently coupled than IP accumulation. In support of this
conclusion, the partial agonist MK212 signaled similarly across all three receptor responses (Figure 5-7 C-D). In fact, the relative efficacies of MK212 and m-CPP were significantly reversed comparing IP accumulation and ERK1/2 phosphorylation (Figure 5-7 D). These data demonstrate for the first time that lisuride and m-CPP are, in fact, functionally selective for ERK1/2 phosphorylation in RSK2+/+ MEFs.

Table 5-4
Relative agonist rank order of efficacy for 5-HT2A-mediated IP accumulation, Ca2+ release, and ERK1/2 phosphorylation in RSK2+/+ and RSK2 -/- MEFs.

<table>
<thead>
<tr>
<th>Rank order</th>
<th>IP accumulation</th>
<th>Ca2+ release</th>
<th>ERK1/2 phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-HT (a)</td>
<td>5-methoxy DMT (a)</td>
<td>Quipazine (a)</td>
</tr>
<tr>
<td>2</td>
<td>α-methyl5-HT (a,b)</td>
<td>Quipazine (a)</td>
<td>5-HT (a,b)</td>
</tr>
<tr>
<td>3</td>
<td>Quipazine (b,c)</td>
<td>DOI (a,b)</td>
<td>Quipazine (a,b,c)</td>
</tr>
<tr>
<td>4</td>
<td>MK212 (c)</td>
<td>5-HT (a)</td>
<td>DOI (a,b,c)</td>
</tr>
<tr>
<td>5</td>
<td>DOI (c,d)</td>
<td>MK212 (b,c)</td>
<td>5-methoxy DMT (b,c,d)</td>
</tr>
<tr>
<td>6</td>
<td>5-methoxy DMT (d)</td>
<td>α-methyl5-HT (c)</td>
<td>MK212 (c,d)</td>
</tr>
<tr>
<td>7</td>
<td>m-CPP (e)</td>
<td>m-CPP (c,d)</td>
<td>m-CPP (a,b,c)</td>
</tr>
<tr>
<td>8</td>
<td>Lisuride (e,f)</td>
<td>Lisuride (d)</td>
<td>Lisuride (e)</td>
</tr>
<tr>
<td>9</td>
<td>SCH-23390 (f)</td>
<td>SCH-23390 (d)</td>
<td>SCH-23390 (e)</td>
</tr>
</tbody>
</table>

*Mean relative efficacies were analyzed via one-way ANOVA and significant differences (significance set as p<0.05) were determined via the Tukey-Kramer multiple comparisons test. Agonists were arranged in descending order (i.e. 1 through 9) and placed into statistically homogeneous groups such that significant differences in rank order are denoted by changes in group membership.
Figure 5-6. Relative rank orders of efficacy were significantly reversed between IP accumulation (●) and ERK1/2 phosphorylation (○) in RSK2+/+ MEFs. Relative efficacy values ($E_{\text{max}}$, 5-HT set to 100%) were determined via nonlinear regression for each agonist (5-HT, DOI, quipazine, 5-methoxyDMT, lisuride, m-CPP, SCH-23390, $\alpha$-methyl5-HT, and MK212) as shown in Tables 5-1 and 5-3. Values represent the mean of four to five independent experiments performed in duplicate.
The Tukey-Kramer multiple comparisons post-test was used to determine significant differences between $E_{\text{max}}$ values for each receptor response as shown in Table 5-4 (*, $p<0.05$). Relative $E_{\text{max}}$ values were significantly increased for the weak partial agonists lisuride and m-CPP at ERK1/2 phosphorylation; whereas the partial agonist MK212 signaled equally in both assays. A significant reversal in relative $E_{\text{max}}$ was observed between m-CPP and MK212.

**Figure 5-7.** Lisuride and m-CPP are functionally selective for ERK1/2 phosphorylation in RSK2+/+ MEFs. A-C, Concentration-response curves for IP accumulation (■), Ca$^{2+}$ release (○), and ERK1/2 phosphorylation (▲) in RSK2+/+ MEFs in response to lisuride (A), m-CPP (B) and MK212 (C). Relative efficacy values ($E_{\text{max}}$, 5-HT set to 100%) were calculated via nonlinear regression as listed in Tables 5-1, 5-2, and 5-3. Values represent the mean +/- SEM of four to five
independent experiments performed in duplicate. D, Statistical analysis of the relative abilities of m-CPP, lisuride, and MK212 to elicit increases in 5-HT$_{2A}$-mediated IP accumulation, Ca$^{2+}$ release, and ERK1/2 phosphorylation in RSK2+/+ MEFs. Significant differences between relative $E_{\text{max}}$ values ($^*$, $p<0.05$) were determined from the Tukey-Kramer multiple comparisons post-test as shown in Table 5-4. Relative $E_{\text{max}}$ values were significantly reversed for m-CPP and MK212 between measures of IP accumulation and ERK1/2 phosphorylation.

5.5 Genetic Deletion of RSK2 Alters Relative Rank Order of Efficacy at 5-HT$_{2A}$ Receptors

By comparing statistically ranked relative efficacies between RSK2+/+ and RSK2/- MEFs I determined that RSK2 deletion substantially changed the relative rank orders of efficacy at all effector readouts (Table 5-4). Further analysis revealed that significant reversals in relative efficacy between RSK2+/+ and RSK2/- MEFs were only observed for agonist-induced IP accumulation (Figure 5-8A, Table 5-4). Specifically, I found that $\alpha$-methyl5-HT signaled similarly to 5-HT in RSK2+/+ MEFs (relative $E_{\text{max}}$=95.4 +/- 1.9% vs. 99.1 +/- 1.2% for $\alpha$-methyl5-HT and 5-HT, respectively, $p>0.05$); whereas 5-methoxyDMT and DOI were ranked significantly lower (relative $E_{\text{max}}$=66.6 +/- 4.0% and 71.7 +/- 2.4% for 5-methoxyDMT and DOI, respectively; $p<0.05$ for both ligands vs. $\alpha$-methyl5-HT) (Figure 5-8B). However, relative efficacies were significantly reversed in RSK2/- MEFs as shown for $\alpha$-methyl5-HT and 5-methoxyDMT (relative $E_{\text{max}}$=134 +/- 3.0% vs. 224 +/- 14% for $\alpha$-methyl5-HT and 5-methoxyDMT, respectively; $p<0.05$), and $\alpha$-methyl5-HT and DOI (relative $E_{\text{max}}$=134 +/- 3.0% vs. 211 +/- 13% for $\alpha$-
methyl5-HT and DOI, respectively; \( p<0.05 \)) (Figure 5-8C). These data show that the relative rank order of efficacy for IP accumulation evolved from \( \alpha \)-methyl5-HT>DOI=5methoxyDMT in RSK2+/+ MEFs to 5-methoxyDMT=DOI> \( \alpha \)-methyl5-HT in RSK2/- MEFs.

Figure 5-8. Relative rank order of efficacy for IP accumulation was significantly reversed between RSK2+/+ (WT, ●) and RSK2/- (KO, ○) MEFs. A, Relative efficacy values (\( E_{\text{max}} \), 5-HT set to 100%) were calculated via nonlinear regression for each agonist (5-HT, DOI, quipazine, 5-methoxyDMT, lisuride, m-CPP, SCH-23390, \( \alpha \)-methyl5-HT, and MK212) as shown in Table 5-1. Values represent the mean +/- SEM of four to five independent experiments performed in duplicate. The Tukey-Kramer multiple comparisons post-test was used to determine significant
differences between $E_{\text{max}}$ values as shown in Table 5-4 (*, $p<0.05$). B-C, Concentration-response curves for IP accumulation in RSK2+/+ (B) and RSK2-/-(C) MEFs following treatment with $\alpha$-methyl5-HT (■), 5-methoxyDMT (○), and DOI (□). Relative efficacy values ($E_{\text{max}}$, 5-HT set to 100%) were determined via nonlinear regression and were significantly potentiated for all agonists in RSK2-/MEFs, as shown in Table 1. Values represent the mean +/- SEM of four to five independent experiments performed in duplicate.

As reported above, variations in $E_{\text{max}}^{\text{RSK2-/-}}/E_{\text{max}}^{\text{RSK2+/+}}$ values indicated that some agonists were differentially responsive to RSK2 deletion, especially when considering 5-HT$_{2A}$-mediated IP accumulation. As seen for $\alpha$-methyl5-HT, 5-methoxyDMT, and DOI, $E_{\text{max}}^{\text{RSK2-/-}}/E_{\text{max}}^{\text{RSK2+/+}}$ values were largely predictive of large shifts in relative efficacy. For example, the partial agonists 5-methoxyDMT and DOI were differentially responsive to RSK2 deletion and exhibited 3.4-fold and 2.9-fold increases in IP accumulation in RSK2-/MEFs, respectively. In contrast, the full agonist $\alpha$-methyl5-HT was the least responsive, resulting in only a 1.4-fold increase in IP accumulation in RSK2-/MEFs. As a result, the relative rank order significantly increased for 5-methoxyDMT and DOI, but not $\alpha$-methyl5-HT, in RSK2-/MEFs. Taken together, the unique responsiveness of 5-methoxyDMT and DOI in the absence of RSK2 accounts for the functional selectivity observed for IP accumulation in RSK2-/MEFs.
5.6 Discussion

Via high-throughput technologies I tested the hypothesis that the signaling of 5-HT$_{2A}$ agonists would be disproportionately affected by changes in RSK2 expression. Specifically, I identified global increases in agonist efficacy, but not potency for 5-HT$_{2A}$-mediated IP accumulation, Ca$_{2+}$ release, and ERK1/2 activation in RSK2/-/- MEFs compared to RSK2+/+ MEFs. These findings imply that 5-HT$_{2A}$ receptors are more responsive in the absence of RSK2 (i.e. less desensitized) and confirms our previous work showing that RSK2 negatively regulates 5-HT$_{2A}$ receptor signaling (172,290). The most intriguing finding of this study was that the patterns of 5-HT$_{2A}$ agonist functional selectivity can be modulated by genetic deletion of a single kinase. This supports the notion that functional selectivity, at least in some cases, results from alterations in the cellular milieu (i.e. conditional efficacy).

In agreement with many studies demonstrating that ligands elicit a gradient of receptor behaviors (for reviews see (6) and (342)), including studies at 5-HT$_{2A}$ receptors (167,330,331), I uncovered novel examples of functional selectivity as follows: 1) between 5-HT$_{2A}$-mediated IP accumulation and ERK1/2 phosphorylation in RSK2+/+ MEFs and 2) between RSK2+/+ and RSK2/-/- MEFs at 5-HT$_{2A}$-mediated IP accumulation.

To expand, I first documented functional selectivity in RSK2+/+ MEFs. This change in relative efficacy could be explained either by increased system responsiveness (i.e. cell-based functional selectivity) or changes in the agonist-receptor complex (i.e. receptor-based functional selectivity). Kenakin (335) has proposed that relative measures of efficacy are system-independent and are solely functions of agonist efficacy. It
follows, then, that a reversal in the relative efficacies of two agonists, a hallmark of receptor-based functional selectivity, requires a change in the agonist-receptor complex (i.e. multiple receptor active states). Consistent with reports of receptor-based functional selectivity for other receptor systems, I found that the relative efficacies of m-CPP and MK212 were significantly reversed between measures of IP accumulation and ERK1/2 phosphorylation.

It is possible that the reversal in relative efficacies between m-CPP and MK212 in RSK2+/+ MEFs could be explained by a single activated receptor state and pathway-specific differences in stimulus-response coupling (e.g. receptor reserve for ERK1/2 phosphorylation). If follows, then, that if stimulus-response coupling was primarily enhanced for one pathway (e.g. ERK1/2) over another (e.g. IP accumulation), I would expect to observe increased efficacy for all partial agonists at the more efficiently-coupled pathway. This assumption is central to the system-independence of the ‘intrinsic efficacy’ concept since the strength of signal imparted to the receptor between two agonists is reflected by the effector response. In functional terms, enhanced stimulus-response coupling manifests as increases in the efficacies of all agonists (i.e. until the response system is saturated) wherein rank order of efficacy is retained, not reversed (320). As presented here, the relative efficacy of the partial agonist MK212 is unchanged comparing measures of IP accumulation and ERK1/2 activation; whereas in the same cells the relative efficacy of m-CPP increased approximately 4-fold for ERK1/2 activation. These data challenge the system-independent notion of intrinsic efficacy. Moreover, these data agree with previous reports of functional selectivity at 5-HT2A.
receptors showing that not all partial agonist efficacies (e.g. quipazine and TFMPP) are increased between two different pathways (330,334).

The second example of functional selectivity at 5-HT$_{2A}$ receptors is evident from a comparison between RSK2+/+ and RSK2/-/- MEFs. These findings show that genetic deletion of RSK2 (i.e. RSK2+/+ vs. RSK2/-/- MEFs) elicits a reversal in relative rank order of efficacy for IP accumulation. Specifically, relative efficacies for IP accumulation were potentiated to different extents in RSK2/-/- MEFs, as illustrated by different $E_{\text{max}}^{\text{RSK2}+/+}/E_{\text{max}}^{\text{RSK2}/-/-}$ values. This suggests that agonists are differentially responsive in cells lacking RSK2 regulation. As above, I argue that the modest differences between RSK2+/+ and RSK2/-/- cell lines (e.g. differences in receptor reserve) cannot account for reversals in relative efficacies since $\alpha$-methyl5-HT is a full agonist in RSK2+/+ MEFs, whereas it was the weakest partial agonist in RSK2/-/- MEFs. Moreover, the inability of $\alpha$-methyl5-HT to signal similarly to the full agonist 5-HT in RSK2/-/- MEFs contradicts the classic intrinsic efficacy model which states that a highly efficacious agonist should signal maximally in a system with increased receptor expression (320).

Conceptually, differences in $E_{\text{max}}^{\text{RSK2}+/+}/E_{\text{max}}^{\text{RSK2}/-/-}$ values and reversals in agonist relative efficacy between RSK2/-/- and RSK2+/+ MEFs are not entirely surprising since auxiliary GPCR interacting proteins, of which there are many (50,51,215), alter ligand activity at target receptors. This new set of pharmacological behaviors is thought to arise from interactions between ligand-enriched GPCR conformations and auxiliary proteins. This phenomenon has been tentatively termed ‘conditional efficacy’. Indeed, our laboratory has previously shown that RSK2 interacts with the 5-HT$_{2A}$ receptor i3 loop.

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(172); although further studies determined that this interaction is insufficient to regulate receptor signaling (290). Instead, RSK2 directly phosphorylates 5-HT$_{2A}$ receptors, thereby attenuating signaling (290). Therefore, it is unlikely that loss of the RSK2-5-HT$_{2A}$ interaction accounts for altered relative efficacy in RSK2-/- MEFs (290).

Alternatively, these data are most consistent with the notion that 5-HT$_{2A}$ agonists are differentially sensitive to RSK2-mediated phosphorylation which maintains the 5-HT$_{2A}$ receptor in a pre-desensitized state (i.e. ‘ionic lock’). Support for pre-desensitized 5-HT$_{2A}$ receptors comes from several key functional experiments showing that genetic deletion of RSK2 leads to significant baseline increases in Ca$^{2+}$ release, inositol phosphate (IP) accumulation, and ERK1/2 phosphorylation (Ryan Strachan and Dr. Bryan L. Roth, unpublished observations). Further support for RSK2-mediated pre-desensitization comes from experiments that measure 5-HT$_{2A}$ signaling over very rapid time frames (seconds) including agonist-induced Ca$^{2+}$ release (peak Ca$^{2+}$ release ~5-10 sec after drug addition) and inositol-1,4,5-trisphosphate (IP$_3$) production (<10 sec). For both readouts, 5-HT$_{2A}$-mediated responses are potentiated in RSK2-/- MEF relative to RSK2+/+ MEFs which suggests that constitutive receptor modification by RSK2 decreases rapid signal transduction events. Interestingly, data from Chapter 4 suggest that receptor pre-desensitization is likely to occur through persistent growth factor activation of RSK2 and subsequent constitutive phosphorylation of Ser 314 within the C-terminal region of the 5-HT$_{2A}$ i3 loop (Chapters 3 and 4)(290). Together, these findings agree with the long held notion that 5-HT$_{2A}$ receptors are not phosphorylated following agonist activation and probably exist in a constitutively phosphorylated basal state ((190,191); Ryan Strachan and Dr. Bryan L. Roth, unpublished observations). This is supported by
several reports from our laboratory showing that 5-HT$_{2A}$ receptor regulation occurs independent of arrestins and GRKs (193,198). Moreover, the fact that 5-HT$_{2A}$ receptors are ‘arrestin-insensitive’ suggests that they are not phosphorylated. In support of this, the ‘arrestin insensitivity’ of 5-HT$_{2A}$ receptors can be reversed upon introduction of a constitutively active arrestin protein that harbors a mutation in the polar core thus mimicking an interaction with a phosphorylated GPCR (200).

Since RSK2-mediated phosphorylation is most likely constitutive and occurs within the C-terminal region of the i3 loop that is important for G$_{a_q}$ coupling and control of 5-HT$_{2A}$ conformational states (35,123), our data could be explained by agonists inducing (or stabilizing) receptor states that exhibit differential sensitivity to Ser314 phosphorylation. It follows, then, that agonist-specific conformations that are heavily regulated by Ser314 phosphorylation in RSK2+/+ MEFs would exhibit a strong potentiation in the absence of RSK2 (e.g. 5-methoxyDMT). Conversely, agonist-specific conformations that are less sensitive to RSK2 regulation would be weakly potentiated in the absence of RSK2 (e.g. $\alpha$-methyl5-HT). Although recent studies have shown evidence of 1) agonist-specific GPCR phosphorylation (343-346), 2) agonist-specific i3 loop conformational changes (347,348), 3) phosphorylation-dependent functional responses (181), and 4) phosphorylation-mediated stabilization of individual receptor conformations (349-351), it remains to be determined how basal levels of phosphorylation differentially affect agonist signaling. Nonetheless, this is an intriguing hypothesis and warrants further study.

In conclusion, a focused screen investigating how expression of the novel GPCR kinase RSK2 modulates 5-HT$_{2A}$ agonist signaling uncovered several major findings.
First, relative agonist efficacy, but not potency, was significantly potentiated for a diverse set of 5-HT$_2$A agonists at measures of IP accumulation, Ca$^{2+}$ release, and ERK1/2 activation in RSK2/- MEFs relative to RSK2+/+ MEFs. Moreover, the magnitude of potentiation derived from $E_{\text{max}}^{\text{RSK2-/-}}/E_{\text{max}}^{\text{RSK2+/+}}$ values was unique for each agonist, thereby suggesting that agonist relative efficacy is differentially regulated by RSK2. Secondly, I identified two novel examples of functional selectivity at 5-HT$_2$A receptors. Statistical analysis of agonist signaling in RSK2+/+ MEFs identified a significant reversal in the relative rank order of efficacy between measures of IP accumulation and ERK1/2 activation. Most intriguingly, I found that RSK2 deletion significantly reversed the relative rank order of efficacy for IP accumulation. To my knowledge this is the first study to demonstrate that deletion of a single kinase promotes differential functional selectivity patterns at a GPCR. Since it is well known that different cell types express distinct sets of GPCR kinases, this study exposes the potential for minor changes in the kinome to produce alterations in effector readouts, with obvious implications for drug actions in vivo.
6.1 Summary

The therapeutic exploitation of the interaction between extracellular ligands and cell surface receptors, which originated as the “drug-receptor” concept, is considered one of the great insights of twentieth-century biomedical science. This statement is indisputable considering that an estimated 5% of the human genome encodes for receptors that mediate physiological responses to a wide range of exogenous and endogenous ligands (9) (i.e. the ‘receptorome’ (352)), and that these receptors account for nearly half of the 500 current therapeutic targets (353). Significantly, a majority of the receptorome is comprised of GPCRs which occupy approximately 2% of the human genome and are known to mediate nearly every physiological process in the body (12). In accord with their vast expression profile and pleiotropic actions, extensive research into the structure and function of GPCRs has yielded a wealth of information that has enhanced our ability to ameliorate human disease as highlighted by recent estimates placing GPCRs as 30% of current marketed small molecule drug targets (354).

GPCR ligands are known to mediate a plethora of functions by acting at numerous GPCRs expressed throughout the body. This is best exemplified by the essential function of serotonin (5-HT), wherein 5-HT binds to at least seven families of receptors (i.e. 5-HT₁₋₇, comprising at least 15 distinct receptors) to influence functions as diverse as cardiovascular regulation, respiration, thermoregulation, circadian rhythm entrainment, sleep-wake cycle, appetite, aggression, sexual behavior, sensorimotor reactivity, pain sensitivity, and learning. Moreover, pharmacological regulation of
serotonin function has been found to influence a range of psychiatric disorders including depression, anxiety, schizophrenia and anorexia nervosa. Also, a range of impulse-related disorders and personality features have been associated with alterations of serotonin function including aggression, substance abuse, gambling, obsessive control, and attention deficit disorder (116). Thus it is clear that 5-HT receptors represent an intriguing yet formidable research focus.

In particular, 5-HT$_{2A}$ receptors are essential for mediating a large number of physiologic processes in peripheral and central tissues including platelet aggregation, smooth muscle contraction, and the modulation of mood and perception. However, 5-HT$_{2A}$ receptor dysregulation has been proposed to be associated with debilitating psychiatric diseases such as schizophrenia. Additionally, most hallucinogens function as 5-HT$_{2A}$ receptor agonists and atypical antipsychotics mediate their actions, at least in part, by antagonizing 5-HT$_{2A}$ receptors. Therefore, it is thought that 5-HT$_{2A}$ receptor activation produces hallucinations, whereas 5-HT$_{2A}$ receptor blockade is therapeutic. From this perspective it is imperative that researchers develop a thorough understanding of the molecular and cellular mechanisms governing 5-HT$_{2A}$ regulation, thereby facilitating a more efficient and focused approach toward treating diseases associated with 5-HT$_{2A}$ receptor dysfunction.

As outlined in Chapter 1, the exact mechanism(s) underlying 5-HT$_{2A}$ receptor regulation remains unclear. In fact, the 5-HT$_{2A}$ receptor is considered an outlier with regard to the canonical GPCR regulation model since it is not detectably phosphorylated after agonist activation and is, in most cell lines, GRK- and arrestin-insensitive. In the thesis presented here, I have made several substantial contributions to our current
understanding of 5-HT\textsubscript{2A} receptor regulation. \textit{First, in Chapter 3} I unambiguously showed that 5-HT\textsubscript{2A} receptor signaling is regulated through a novel mechanism whereby the downstream ERK effector RSK2 directly phosphorylates Ser314 situated within the third intracellular loop (i3 loop) (290). This finding is important since it 1) directly confirms the previous report by Sheffler et al. (172) showing that RSK2 interacts with and regulates 5-HT\textsubscript{2A} receptor signaling, 2) it provides the first evidence that 5-HT\textsubscript{2A} receptors are phosphorylated by a specific kinase and that this phosphorylation event is associated with deficits in signal transduction, and 3) it provides direct evidence for cross-talk between a downstream effector of the ERK/MAPK pathway and a GPCR. Significantly, the identification of a \textit{bona fide} 5-HT\textsubscript{2A} phosphorylation site with functional consequences directly answers the enduring question of whether or not 5-HT\textsubscript{2A} receptors are regulated by direct phosphorylation. Moreover, these findings represent a methodological achievement for 5-HT\textsubscript{2A} research considering the difficulties inherent to the purification and mass spectrometry of integral membrane proteins.

\textit{Second, in Chapter 4} I employed several experimental approaches to show that cross-talk between growth factor-activated receptor tyrosine kinases (RTKs) and several GPCRs requires RSK2. These results are the first to implicate a downstream ERK/MAPK pathway effector in RTK-mediated heterologous desensitization of GPCRs. Moreover, they are significant to the 5-HT\textsubscript{2A} field since they imply that 5-HT\textsubscript{2A} receptor activity is modulated by growth factor signaling. This is an important finding considering the association between aberrant growth factor signaling and human disease such as schizophrenia (discussed below).
Third, in Chapter 5 I showed that 5-HT$_{2A}$ receptor agonists display selectivity for one receptor response over another (i.e. functional selectivity), and that the absence or presence of RSK2 has differential effects on 5-HT$_{2A}$ agonist signaling. These results further support the conceptual framework of functional selectivity wherein agonists exhibit a range of efficacies for different receptor responses. Most importantly, I observed that RSK2 expression differentially modulated agonist signaling which led to changes in the pattern of functional selectivity. These results show for the first time that a relatively minor change in the cellular kinome profoundly affects ligand behavior (i.e. conditional efficacy) and suggests that agonists exhibit differential sensitivity to receptor phosphorylation.

6.2 Implications for Future Studies

Examining my results from a larger perspective, the results presented in Chapters 3, 4, and 5 of this thesis support a novel regulatory mechanism whereby RTKs act via RSK2 to desensitize GPCR signaling. Mechanistically, as presented here for the 5-HT$_{2A}$ receptor, growth factor activation of some RTKs (e.g. epidermal and platelet-derived growth factor receptors, EGF receptor and PDGF receptor, respectively) promotes canonical activation of the downstream multifunctional ERK/MAPK effector RSK2, which then interacts with and directly phosphorylates the 5-HT$_{2A}$ receptor within the i3 loop to decrease receptor responsiveness. This novel mechanism is a significant departure from the canonical model of GPCR regulation since it extends the notion of heterologous desensitization to include a regulatory input from a seemingly distinct signal transduction pathway. Moreover, 5-HT$_{2A}$ receptors are probably regulated via
alternative, non-canonical pathways since it is clear that 5-HT2A receptors are not phosphorylated following agonist treatment and have been shown to be GRK- and arrestin-insensitive. The data presented here imply 1) that the C-terminus of the 5-HT2A receptor i3 loop is important for G protein coupling since phosphorylation in this region is associated with altered 5-HT2A receptor signaling, 2) that 5-HT2A receptor signaling is typically modulated by growth factor-activation of RTKs and may explain how 5-HT2A receptors are regulated in vivo, and 3) that phosphorylation of the 5-HT2A receptor differentially affects agonist signaling thereby promoting changes in functional selectivity patterns. Several studies have been proposed to answer each of these questions and will be detailed below.

6.2.1 The C-Terminus of the 5-HT2A Receptor I3 Loop is Important for Regulating G Protein-Mediated Signaling

As presented in Chapter 3, I showed that RSK2 directly phosphorylates Ser314 located within the C-terminal region of the 5-HT2A receptor i3 loop. This implies that Ser314, and perhaps other regions of the i3 loop, are important for modulating receptor signaling. However, the mechanism(s) underlying this modulatory effect remain unknown. As discussed in Chapter 3 several studies assessing G protein activation have provided some insight into this mechanism. For instance, Oksenberg et al. (315) showed that the i3 loop of 5-HT receptors is important for G protein coupling since introduction of the i3 loop of the Gaq-coupled 5-HT2A receptor into the Gai-coupled 5-HT1B receptor (5-HT1B/2A) is sufficient to shift its coupling specificity from inhibition of adenylyl cyclase to PLC activation. Moreover, previous work in our lab suggests that peptides
corresponding to the C-terminal regions of the 5-HT$_{2A}$ i3 loop directly interact with and activate purified Gaq (123). Also, it is known that mutating Cys322 (Cys6.34) within the C-terminal region of the i3 loop leads to constitutively active 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors (35), as replicated by others (355). Some further insight into the mechanism underlying the modulatory effect of Ser314 phosphorylation also comes from structural studies. One such study conducted by Gelber et al. (199) demonstrated that the purified 5-HT$_{2A}$ receptor i3 loop is predominantly α-helical and binds purified arrestins. Although the exact site of arrestin binding was not determined, it is intriguing that the i3 loop can bind the canonical regulatory protein arrestin, whose binding is enhanced by phosphorylation. Also, these findings agree with several studies suggesting that amphipathic α-helices are involved in determining receptor-G protein binding (313,314) and may, in fact, be required for Gaq coupling as suggested by the recent structure of Gaq-coupled squid rhodopsin (25). Together with previous studies showing that introduction of phosphorylated residues into the cytoplasmic domains of many GPCRs inhibits signaling, it seems likely that Ser314 phosphorylation could uncouple the 5-HT$_{2A}$ receptor from its cognate G protein.

Although plausible, the hypothesis that Ser314 phosphorylation uncouples the 5-HT$_{2A}$ receptor from Gaq is highly speculative and requires additional tests. Several of these tests are outlined here. First, functional experiments incorporating mutations that mimic Ser314 phosphorylation (i.e. phosphomimetic, typically Asp or Glu) would be very informative. Although not presented here, these studies have been previously performed as part of the thesis work of Dr. John A. Gray. In these studies Dr. John A. Gray introduced wild-type and S314D phosphomimetic mutant 5-HT$_{2A}$ receptors into
HEK 293 cells and measured 5-HT-induced IP accumulation. In support of the uncoupling hypothesis, his preliminary data showed that the phosphomimetic receptors exhibited decreased 5-HT maximal efficacy compared to wild-type receptors (Dr. John A. Gray, unpublished results). These preliminary data are exciting since they both highlight this region as important for G protein coupling and suggest that the introduction of a phosphate group is sufficient to uncouple the receptor. Future functional studies (e.g. Ca\(^{2+}\) release and IP accumulation) aimed at replicating these findings in RSK2-/- MEFs should prove to be very informative. It is important to note that these experiments should be conducted in RSK2-/- MEFs thus ensuring that the wild-type receptor is not phosphorylated. In addition to second messenger studies, a co-immunopurification approach similar to the one employed by Bhatnagar et al. (171) would measure the propensity for the S314D phosphomimetic mutant receptor to couple to G protein. In this approach, analyzing the amount of G\(_{\alpha q}\) that co-purifies with wild-type or phosphomimetic 5-HT\(_{2A}\) receptors from RSK-/- fibroblasts would indirectly measure the effect that 5-HT\(_{2A}\) receptor phosphorylation has on G protein coupling. Aside from indirect methods, the use of Surface Plasmon Resonance (SPR) would directly measure the i3 loop-G\(_{\alpha q}\) interaction. In the SPR method proposed here, purified G\(_{\alpha q}\) would be immobilized on an SPR surface and then various concentrations of purified wild-type i3 loop peptide would be used to measure the affinity of the i3 loop-G\(_{\alpha q}\) interaction. If these initial experiments show an interaction, then determining the effect that Ser314 phosphorylation has on coupling could be directly tested by incorporating either a phosphomimetic mutant i3 loop peptide or a wild-type i3 loop peptide that has been phosphorylated in vitro by RSK2 and then re-purified. Although the latter approach is
more laborious, it would directly measure any changes in affinity resulting from Ser314 phosphorylation. Applying the phosphomimetic approach to complementary approaches such as GTPγS binding and radioligand competition assays (i.e. [3H]-ketanserin vs. DOI) would also determine if introduction of a phosphate into this region affects G protein coupling.

In Chapter 3 I proposed an alternative mechanism whereby Ser314 could strengthen the ‘ionic lock’ between TMs 3 and 4, thereby promoting an inactive receptor conformation. Several key observations supported this hypothesis. First, our laboratory has previously shown that disruption of this ‘ionic lock’ by mutating Glu318 (6.30) to Arg increases constitutive activity and agonist affinity of the 5-HT2A receptor (35). In addition, Ser314 (Ser6.26) is located one helical turn down from the highly conserved Glu318 (Glu6.30) which serves as the counter ion to Arg3.50. This places Ser314 on the same helical face as Glu318 and upon phosphorylation might favor an electrostatic interaction with Arg3.50. Although speculative, structure-based experiments could test this alternative explanation. Specifically, microsecond time-scale molecular dynamics simulations have been recently used to specifically interrogate changes in the ‘ionic lock’ of wild-type and mutant β2ARs and could be directly applied to wild-type and mutant (phosphomimetic, S314D) 5-HT2A receptors (356). In simulations of wild-type constructs the authors found that the ionic lock was preferred, whereas in constitutively active receptors an equilibrium was established that favored a broken ionic lock. These data are consistent with biochemical evidence suggesting that the ionic lock stabilizes the inactive state of GPCRs (35,357). It follows that if the hypothesis is true, one would expect to
observe an increase in conformations adopting the ‘ionic lock’ in the S314D mutant receptors.

6.2.2 RSK2 is Required for the RTK-Mediated Heterologous Desensitization of 5-HT$_{2A}$ Receptor Signaling \textit{In Vivo}

The data I presented in \textbf{Chapter 4} demonstrated that RSK2 is required for RTK-mediated heterologous desensitization of 5-HT$_{2A}$ receptors. Specifically, 5-HT$_{2A}$ responsiveness is decreased following activation of EGF and PDGF receptors, but not the insulin receptor, in a variety of cells expressing RSK2. Moreover, I showed that P2Y-purinergic signaling was decreased following EGF receptor activation in a RSK2 dependent manner and is consistent with its ability to be regulated by RSK2 (172). Importantly, considering the essential functions of RTKs in many cell types, these findings imply that RTK-mediated heterologous desensitization of GPCRs represents a conserved regulatory mechanism in cells. The best support for this mechanism comes from the functional antagonism of glucose homeostasis in which both insulin and the insulin-like growth factor-1 inhibit β$_1$- and β$_2$AR signaling. This cross-regulatory effect of insulin on βAR-mediated cAMP accumulation has physiological implications since catecholamines act in opposition to insulin by stimulating glycogen breakdown, gluconeogenesis, and lipolysis (114).

Likewise, it is tempting to speculate that RTK-mediated heterologous desensitization of GPCRs occurs in the brain and is important for regulating 5-HT$_{2A}$ receptors. For instance, a diverse literature shows that RTK ligands including the neurotrophins (i.e. brain-derived neurotrophic factor, BDNF and nerve growth factor,
NGF) and neuregulins (i.e. neuregulin-1, NRG-1) play essential roles in the developing and mature brain (322,323). During development, neurotrophins have been shown to regulate naturally occurring cell death, synaptic connectivity, fiber guidance, and dendritic morphology. In the mature brain neurotrophins contribute to brain plasticity since they are involved in activity-dependent neuronal function. Similar to the functions attributed to neurotrophins, NRG-1 is also associated with neural development as well as control of synaptic plasticity and neuronal survival. In fact, the neuregulin receptor (ErbB4) is expressed throughout the mature brain and is known to reside in cortical layers 2-6b (323). Moreover, ErbB4 is known to interact with PSD-95, a post-synaptic density protein that associates with and regulates 5-HT_{2A} receptor signaling and trafficking in vitro and in vivo (209,213,214,358). Thus, considering the central role of 5-HT_{2A} receptor signaling in the modulation of mood and perception, and its expression throughout the brain including enrichment in cortical layer 5, the potential for regulation by RTKs is a highly intriguing concept. Most importantly, aberrant signaling of both RTKs and 5-HT_{2A} receptors has been associated with neuropsychiatric disorders such as depression and schizophrenia (123,323,324,359). Therefore, a more thorough understanding of the mechanism(s) underlying RTK-mediated regulation of 5-HT_{2A} receptor signaling could provide us with more effective therapeutic targets.

Here I propose to explore RTK-mediated heterologous desensitization of 5-HT_{2A} receptors in a physiologically relevant context by determining if growth factor pre-treatment leads to decreased 5-HT_{2A} responsiveness in ex vivo cultured cortical neurons. These experiments would incorporate the standard isolation and culturing of cortical neurons (postnatal day 1) from both RSK2+/+ and RSK2/- mice and live cell Ca^{2+}
imaging that has been developed in our laboratory (Chapter 2 MATERIALS AND METHODS). As demonstrated in preliminary experiments recently performed in the laboratory by Dr. John Allen, we can reproducibly measure the Ca$^{2+}$ response elicited by the selective 5-HT$_2A$ agonist DOI in cultured cortical neurons (Figure 6-1A). Most importantly, a 1 hr pre-treatment with 100 ng/mL EGF and NRG1 significantly decreased the responsiveness of 5-HT$_2A$ receptors in RSK2+/+ neurons (Figure 6-1B, Table 6-1). At first glance these data confirm our results in cultured cells and suggest that, indeed, growth factors regulate 5-HT$_2A$ receptors in neurons. We are currently replicating these results and determining in parallel if RSK2 is required for RTK-mediated heterologous desensitization of 5-HT$_2A$ receptors. Additional experiments incorporating the phosphorylation-deficient 5-HT$_2A$ mutant (S314A) will be required to directly determine if Ser314 phosphorylation mediates this effect. These data are extremely exciting and have enormous potential for explaining how 5-HT$_2A$ receptors are regulated in vivo.
Figure 6-1. Neuronal 5-HT$_{2A}$-mediated calcium signaling is densensitized by epidermal growth factor (EGF) and neuregulin-1 (NRG-1). Primary cortical neurons were isolated and cultured as detailed in Chapter 2 MATERIALS AND METHODS. After 7 days in culture, neurons were infected with lentivirus containing 5-HT$_{2A}$-GFP-CT cDNA (GFP introduced within C-terminus, see (213)). Forty-eight hours after infection and expression, neurons were loaded with calcium dye and pretreated for 1 hr with 100 ng/ml EGF or neuregulin-1. After growth factor treatments, neurons were stimulated with 1 µM DOI and calcium dye fluorescence was assessed using high content imaging fluorescence microscopy. A, Representative images of DOI-induced calcium dye fluorescence in cortical neuron cultures expressing 5-HT$_{2A}$-GFP-CT show that 10 µM DOI addition resulted in
increased calcium dye fluorescence (compare left and middle panels). Responding cells were segmented (right panel) and fluorescence intensity was quantified and normalized to GFP fluorescence in each well. B, Mean (+/- SEM) of DOI-induced calcium dye fluorescence in uninfected cells, 5-HT$_{2A}$-GFP-CT expressing neurons, or 5-HT$_{2A}$-GFP-CT expressing neurons pre-treated with growth factor (N=2 neuronal preps and $\geq$100 cells analyzed per group). These preliminary studies performed by John Allen indicate that both EGF and NRG-1 significantly desensitize 5-HT$_{2A}$ receptors in cultured cortical neurons.

Table 6-1

**Effects of EGF and NRG-1 on DOI-induced Ca$^{2+}$ release in RSK2+/+ neurons.**

<table>
<thead>
<tr>
<th></th>
<th>Peak Ca$^{2+}$ response</th>
<th>Area Under Curve</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1uM DOI</td>
<td>0.72 ± 0.04</td>
<td>39.6 ± 1.3</td>
<td>60</td>
</tr>
<tr>
<td>100ng/ml NRG1 + DOI</td>
<td>0.67 ± 0.06</td>
<td>34.3 ± 1.9 *</td>
<td>69</td>
</tr>
<tr>
<td>100ng/ml EGF + DOI</td>
<td>0.51 ± 0.04 *</td>
<td>24.9 ± 1.5 *</td>
<td>63</td>
</tr>
</tbody>
</table>

Data are pooled results from two independent cell preps (mean +/- SEM), N is total number of cells; *, p<0.05 vs. DOI
6.2.3 Phosphorylation of the 5-HT\textsubscript{2A} Receptor Differentially Affects Agonist Signaling Thereby Promoting Altered Patterns of Functional Selectivity

The findings presented in Chapter 5 both support the emerging concept of functional selectivity and extend it to include contributions by the cellular kinome. First, the evidence of functional selectivity in RSK2+/+ MEFs suggests for the first time that lisuride and m-CPP are functionally selective for ERK1/2 phosphorylation. These data confirm previous studies that identified functional selectivity at 5-HT\textsubscript{2A} receptors (167,330,331). The fact that ligands are functionally selective has profound implications for drug discovery. Namely, it changes the perception of what proper drug target validation should be and suggests that not only should the receptor’s involvement in pathological and/or therapeutic processes be validated, but also as much information about the pertinent signaling pathways involved should be determined. In this way, monitoring the activation of the most relevant signaling pathways, either by performing single experiments or multiplexing assays through the use of techniques such as high content microscopy, could then identify molecules that offer better therapeutic efficacies but lack unwanted side effects (360).

Second, I showed that in addition to functional selectivity in MEFs, genetic deletion of RSK2 resulted in a significant reversal in the relative rank order of efficacy for 5-HT\textsubscript{2A} agonists. As one might expect, these findings have important implications for drug discovery since slight changes in the cellular milieu can elicit large changes in relative agonist efficacies (i.e. conditionally efficacious ligands). Significantly, this highlights the importance of choosing appropriate cell lines for small molecule screening and portends the use of complex primary cell-based models.
In Chapter 5 I suggested that the conditional efficacy observed when comparing RSK2+/+ and RSK2/- MEFS could be explained by the differential sensitivity of agonists to RSK2 regulation, as indicated by variations in $E_{\text{max}}^{\text{RSK2-}/+}/E_{\text{max}}^{\text{RSK2+/+}}$. This implies that agonists are differentially modulated by receptor phosphorylation and is supported by studies showing that phosphorylation events can differentially alter receptor conformations (343-346,349; Francesconi, 2000 #795; Palanche, 2001 #580). However, it remains to be determined how receptor phosphorylation differentially affects agonist signaling. Some insight comes from recent studies suggesting that agonists promote specific i3 loop conformational changes (347,348). Therefore, it is possible that some agonist-promoted conformations are more sensitive than others to Ser314 phosphorylation. This hypothesis could be tested using several different methodologies. For example, measuring the relative ability (i.e. compared to 5-HT) of each agonist to activate wild-type and phosphomimetic (S314D) mutant 5-HT$_{2A}$ receptors expressed in RSK2/- MEFS would test the sensitivity of each agonist to the phosphorylation event. A complementary approach would involve measuring affinity changes in wild-type and phosphomimetic (S314D) mutant receptors via radioligand binding experiments (i.e. agonist vs. [$^3$H]-ketanserin). Given that agonists displayed differential sensitivity to RSK2 regulation, I would predict that agonists that were highly responsive in RSK2/- MEFS (i.e. large $E_{\text{max}}^{\text{RSK2-}/+}/E_{\text{max}}^{\text{RSK2+/+}}$ values) would show the greatest change in affinity at the phosphomimetic mutant receptor. For instance, the high affinity binding component of $\alpha$-methyl5-HT should be modestly decreased in both assays (i.e. it is less sensitive to RSK2 regulation); whereas, the affinity changes for 5-methoxyDMT should be significantly larger as evidenced by its robust potentiation in RSK2/- MEFS (i.e. more
sensitive to RSK2 regulation). In addition to explaining how RSK2 deletion promotes changes in the patterns of functional selectivity, these experiments will provide further insight into the exact mechanism whereby RSK2-mediated phosphorylation of 5-HT$_{2A}$ receptors at Ser314 regulates signaling.

### 6.2.4 RSK2 Regulates 5-HT$_{2A}$ Receptors In Vivo.

The ultimate goal of basic science research is to develop a detailed understanding of the mechanisms underlying fundamental processes in the human body. In doing so, the expectation is that we can more effectively ameliorate dysfunctions in these fundamental processes which are associated with human disease. As presented in this thesis, I have made new contributions to our understanding of the mechanism whereby RSK2 regulates 5-HT$_{2A}$ receptor signaling in cultured cells. Importantly, these findings led to the discovery of a novel regulatory paradigm in which RSK2 is required for RTK-mediated negative regulation of GPCRs including the 5-HT$_{2A}$ and P2Y-purinergic receptors. Considering the importance of growth factor signaling for basic cellular functions throughout the body, these data imply that RSK2 is an important regulator of GPCR signaling in vivo. Moreover, reports correlating RSK2 mutations in humans (i.e. Coffin-Lowry syndrome) with a schizophrenia-like psychosis suggest that the role of RSK2 in 5-HT$_{2A}$ function in vivo does, indeed, warrant further study (283).

Several experimental approaches could directly test the hypothesis that RSK2 regulates 5-HT$_{2A}$ receptor signaling in vivo. First, identical to the approach outlined above in Chapter 6.2.3 (also Chapter 2 MATERIALS AND METHODS), 5-HT$_{2A}$-mediated Ca$^{2+}$ release in cultured cortical neurons isolated from RSK2+/+ and RSK2/
mice could be directly compared. I would predict that similar to observations in RSK2-/mice, 5-HT2A-mediated Ca\(^{2+}\) signaling would be significantly potentiated in the RSK2-/cortical neurons. In a second, more relevant approach, I propose the use of RSK2-/ mice. These mice have been used previously to examine the role of RSK2 in skeletal abnormalities (246), in learning and memory (285,361), in glycogen metabolism (361), in the regulation of body weight, adipose mass, and glucose homeostasis (362), and in neurotransmission (363). Likewise, subjecting these animals to a battery of behavioral tests designed to measure 5-HT2A receptor function \textit{in vivo} would interrogate the role of RSK2 in 5-HT2A receptor function. The most reliable of these tests is the head twitch response (HTR) which has been shown by Gonzalez-Maeso et al. (364) and others (365) to be mediated exclusively by 5-HT2A receptors. Additional tests including the phencyclidine (PCP)-induced disruption of pre-pulse inhibition (PPI) and PCP-induced hyper locomotion-both of which are blocked by 5-HT2A receptor antagonists-could be used to interrogate 5-HT2A receptor activity \textit{in vivo} (366-368).

In preliminary behavioral experiments (Figure 6-2A), significant differences in motor activity between RSK2+/+ and RSK2-/ mice were not observed, in conformation of a prior report (285). This confirms the suitability of RSK2-/ mice for behavioral testing. In addition, preliminary analysis of DOI-induced HTR showed a dose-dependent increase in the number of head twitches over 60 min. Surprisingly, the total number of head twitches was consistently lower in RSK2-/ mice when compared to wild-type littermate controls at each dose of DOI and was statistically different at 0.75 mg/kg DOI (141 +/- 9 vs. 119 +/- 12 total head twitches in 60min for RSK2+/+ and RSK2-/ mice, respectively; N=9-10 animals per genotype, \(p<0.05\)) (Figure 6-2B). As shown in Figure
6-2C, the HTR elicited by 0.75 mg/kg DOI was maximal by 15-20 min and was significantly lower in RSK2/- mice during that interval. Although these data are inconsistent with the *a priori* prediction that RSK2 behavior would be increased in the RSK2/- mice relative to the RSK2+/+ mice, they are nonetheless intriguing and provide the first *in vivo* evidence suggesting that RSK2 plays a role in regulating RSK2 in an intact animal. Additional behavioral tests including (PCP)-induced disruption of pre-pulse inhibition (PPI) and PCP-induced hyper locomotion are currently being implemented to further explore the *in vivo* regulation of 5-HT2A signaling by RSK2.
Figure 6-2. Preliminary behavioral experiments comparing 5-HT₂A signaling in RSK2+/+ and RSK2−/− mice. A, Exploratory activity in a novel environment was assessed by 1 hr trials, given on separate days, in an open field chamber (40 cm x 40 cm x 30 cm) crossed by a grid of photo beams (VersaMax system, AccuScan...
Data shown are means +/- SEM taken at 5 min intervals for 6 RSK2+/+ and 6 RSK2-/- mice. No significant differences were found between the RSK2+/+ and RSK2-/- mice for any measures taken during the activity test. B and C, The head twitch response (HTR) elicited by various doses of DOI (i.e. 0.1, 0.3, 0.75, and 1.0 mg/kg) in 10 RSK2+/+ and 9 RSK2-/- mice were manually scored by blinded observers every 5 min over the course of 1 hr. As presented in (B) the total number of head twitches scored in drug naïve mice were subtracted from the total number of twitches scored for each dose of DOI over 1 hr and presented as means +/- SEM. RSK2+/+ mice consistently scored higher at all doses of DOI when compared to RSK2-/- mice and were significantly different at 0.75 mg/kg DOI, \( p<0.05 \). Plotting the means +/- SEM vs. time in (C) shows that the maximal HTR elicited by 0.75 mg/kg DOI was significantly higher in RSK2+/+ mice when compared to RSK2-/- mice. These preliminary data suggest that genetic deletion of RSK2 leads to altered 5-HT_{2A} signaling.

6.3 Closing Considerations

RSK2 was initially identified by Dr. Douglas J. Sheffler and Dr. Wesley Kroeze in a yeast-two-hybrid screen that identified several 5-HT_{2A} receptor i3 loop interacting proteins. RSK2 was subsequently selected for characterization based upon 1) its role as a downstream kinase effector of ERK/MAPK that typically phosphorylates serine residues within a basophilic consensus motif similar to the one located within the i3 loop, and 2) its role in the rare syndromic form of X-linked mental retardation known as Coffin-Lowry syndrome which sometimes manifests as a schizophrenia-like psychosis. Progressing from initial studies verifying that RSK2 interacts with 5-HT_{2A} receptors in
and in vivo, it was later determined that RSK2 negatively regulates the signaling of many GPCRs including 5-HT2A receptors, perhaps via direct phosphorylation. In the approach discussed herein, I unequivocally demonstrate that activated RSK2 phosphorylates 5-HT2A receptors at Ser314 in vitro and in intact cells, thereby modulating its signaling. Indeed, I showed that this phosphorylation event differentially modulates agonist signaling as evidenced by extreme cases of functional selectivity when RSK2 expression is lost. These data provide the first direct link between 5-HT2A receptor phosphorylation and regulation, and considering that the mechanism(s) underlying 5-HT2A regulation are largely unknown, will have a tremendous impact on the 5-HT2A receptor field. Furthermore these initial findings ultimately led to the discovery of a novel regulatory mechanism whereby activated RTKs, in addition to their classical modulation of mitogenic signaling cascades, were acting through RSK2 to heterologously desensitize GPCRs. Importantly, these data are the first to show that RSK2 is required for RTK-GPCR cross-talk and suggest that RTKs are likely to regulate 5-HT2A receptors in vivo. This novel mechanism is supported by recent evidence showing that RTK-GPCR cross-talk is physiologically relevant, as shown for β1-, β2-, and α1bAR regulation by insulin. Indeed, our preliminary findings in cultured cortical neurons suggest that RTK activation desensitizes 5-HT2A receptors, thereby setting the stage for future discoveries.

In closing, this thesis work is the result of a rare opportunity afforded to me by the careful groundwork of Dr. Douglas J. Sheffler and intellectual guidance of Dr. Bryan L. Roth. Specifically, the multidisciplinary approach detailed in this thesis has advanced RSK2 from humble beginnings as an unknown 5-HT2A interacting protein to a novel GPCR kinase that functions as a regulatory node between growth factor-activated RTKs
and GPCRs. Given this trajectory, further inquiry into the regulation of GPCRs is destined to yield fascinating results.
BIBLIOGRAPHY


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