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INSIGHTS INTO THE REGULATION OF 5-HT_{2A} RECEPTORS BY SCAFFOLDING PROTEINS AND KINASES

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SUMMARY

5-HT_{2A} serotonin receptors are essential molecular targets for the actions of LSD-like hallucinogens and atypical antipsychotic drugs. 5-HT_{2A} serotonin receptors also mediate a variety of physiological processes in peripheral and central nervous systems including platelet aggregation, smooth muscle contraction, and the modulation of mood and perception. Scaffolding proteins have emerged as important regulators of 5-HT_{2A} receptors and our recent studies suggest multiple scaffolds exist for 5-HT_{2A} receptors including PSD95, arrestin, and caveolin. In addition, a novel interaction has emerged between p90 ribosomal S6 kinase and 5-HT_{2A} receptors which attenuates receptor signaling. This article reviews our recent studies and emphasizes the role of scaffolding proteins and kinases in the regulation of 5-HT_{2A} trafficking, targeting and signaling.

Keywords

5-HT_{2A}; 5-hydroxytryptamine; serotonin; PSD95; arrestin; caveolin; lipid raft; RSK2; PKC; GPCR; Gq; PDZ; post-synaptic density; desensitization; trafficking; endocytosis; internalization; scaffolding

INTRODUCTION

The 5-hydroxytryptamine (serotonin) receptor 2A (5-HT_{2A}) is an important G protein-coupled receptor (GPCR) that has been implicated in many psychiatric disorders including schizophrenia, mood disorders, anxiety disorders, obsessive-compulsive disorder, eating disorders, and Alzheimer's disease (Abbas and Roth, 2008; Roth et al., 1998b; Roth and Xia, 2004). 5-HT_{2A} receptors are essential for mediating many physiological processes including platelet aggregation, smooth muscle contraction and the modulation of mood and perception (Roth et al., 1998a). Since dysregulation of the 5-HT_{2A} serotonergic system has been implicated in a large number of diseases, serotonin receptors have become molecular targets for multiple drugs of diverse therapeutic classes. 5-HT_{2A} receptors are the site of action of most (Aghajanian, 1994; Glennon, 1990; Nichols, 2004; Roth et al., 2002), but not all (Roth et al., 2002; Sheffler and Roth, 2003) hallucinogens including lysergic acid diethylamide, psilocybin and mescaline—all of which function as 5-HT_{2A} receptor agonists. The 5-HT_{2A}

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receptors are also principle molecular targets for atypical antipsychotic drugs as well as targets of antidepressants, and anxiolytics (Gonzalez-Maeso et al., 2007; Gray and Roth, 2007; Kroeze et al., 2003). Given that 5-HT_{2A} receptors play crucial roles in the modulation of perception, cognition, and emotion (Jakab and Goldman-Rakic, 1998; Kroeze and Roth, 1998; Roth, 1994; Roth et al., 1999), determining the molecular and cellular mechanisms governing 5-HT_{2A} function may provide insights into the pathogenesis of psychiatric diseases including the pathophysiology of schizophrenia and depression.

The 5-HT_{2A} receptor is widely expressed in the central nervous system; however, it is the most abundant serotonin receptor in cerebral cortex where it is enriched in apical dendrites and dendritic spines of cortical pyramidal neurons of layers IV and V (Miner et al., 2003; Willins et al., 1997; Xia et al., 2003b). Since the molecular cloning of the 5-HT_{2A} receptor there has been steady progress in understanding the molecular biology (Roth et al., 1998a), protein structure (Roth and Shapiro, 2001), and intracellular trafficking of the receptor (Gray and Roth, 2001). A number of scaffolding proteins have emerged as important regulators of 5-HT_{2A} receptors (Becamel et al., 2004; Bhatnagar et al., 2004; Cornea-Hebert et al., 2002; Gelber et al., 1999; Schmid et al., 2008; Sheffler et al., 2006; Xia et al., 2003a; Xia et al., 2003b). These scaffolding interactions likely serve multiple roles and primarily regulate receptor function by influencing the subcellular localization of the receptors (Figure 1). This paper reviews several recent advances emphasizing the role of scaffolding proteins and kinases in the control of 5-HT_{2A} trafficking, targeting and signaling.

Scaffolding proteins and 5-HT_{2A} receptors: relevance for synaptic targeting, trafficking, and signal transduction

PSD95 and the subcellular targeting of 5-HT_{2A} receptors—Protein interaction motifs, such as PDZ, SH2 and SH3 domains, mediate protein-protein interactions by recognizing short peptide epitopes within their interacting partners (Castagnoli et al., 2004; Kuriyan and Cowburn, 1997; Sudol, 1998). The PDZ domain, roughly 90 amino acids long, is one of the best characterized classical protein interaction motifs. The PDZ domain was first discovered as sequence repeats in the primary structures of the post-synaptic density 95 (PSD95), disk-large (Dlg) and zona occludens-1 (ZO-1) proteins (Cho et al., 1992). Several PDZ domain proteins, particularly those containing multiple PDZ motifs, function as scaffolds at specialized membrane regions in the cell, where they regulate the organization and maintenance of large molecular complexes, such as signal-transduction machinery at post-synaptic densities (Kennedy, 2000; Kim and Sheng, 2004). Moreover, by providing a scaffold these proteins link various components of signal transduction pathways that regulate the speed and specificity of signaling.

PSD95 interacts with variety of protein substrates such as NMDA type glutamate receptors (Kornau et al., 1995), β 1-adrenergic receptor (Hu et al., 2000), neuroligin (Irie et al., 1997), and citron (Zhang et al., 1999), although the functional relevance of these interactions has only been recently appreciated (Mendoza-Topaz et al., 2008). Using a proteomic approach, the 5-HT_{2C} serotonin receptor was the first serotonin receptor family member shown to interact with PSD95 *in vivo* (Becamel et al., 2002). Although a PSD95 interaction with the 5-HT_{2A} receptor was predicted based on sequence homology and *in vitro* binding with purified carboxy-terminal peptides, the first specific interaction of PSD95 with 5-HT_{2A} receptors was demonstrated in HEK293 cells (Xia et al., 2003a). In this study, using confocal microscopy and coimmunoprecipitation studies, it was shown that 5-HT_{2A} receptor shares a canonical Type I PDZ-binding domain (X-Ser/Thr-X- ϕ : SCV) and mutation or deletion of this PDZ-binding domain completely disrupts the PSD95 interaction, confirming the specificity of this association. Furthermore, co-transfection of PSD95 and wild-type 5-HT_{2A} significantly enhanced serotonin-induced inositol phosphate accumulation, highlighting the functional

importance of PSD95 interaction for 5-HT_{2A} signaling. Interestingly, PSD95 does not modulate constitutive 5-HT_{2A} receptor signaling nor the kinetics of agonist dependent 5-HT_{2A} receptor desensitization. It was not surprising that the PDZ-binding motif was not required for agonist-induced 5-HT_{2A} receptor desensitization, as mutation of the PDZ domain motif did not prevent 5-HT_{2C} receptor desensitization (Backstrom et al., 2000). Considering all the experimental evidence, it is quite conceivable that PSD-95 provides a scaffolding platform for downstream signaling molecules such as Gq and PLC, and thus facilitates enhanced signaling by the 5-HT_{2A} receptor. However, the potentiation of 5-HT_{2A} receptor signaling by PSD95 binding in HEK293 cells warrants further validation in more relevant experimental systems such as primary neuronal cultures and *in vivo* studies.

Furthermore, it was shown that PSD-95 attenuates agonist-mediated 5-HT_{2A} receptor internalization (Xia et al., 2003a), possibly due to the recruitment and anchoring of multiple proteins to the plasma membrane, creating a macromolecular complex between PSD95 and the 5-HT_{2A} receptor that cannot be internalized (Figure 2). The established paradigm for GPCR internalization is agonist-induced phosphorylation of receptors mediated by G protein-coupled receptor kinases (GRK) followed by internalization through clathrin-coated pits (Gray and Roth, 2002). In such a scenario, one can assume that if PSD95 binding to 5-HT_{2A} receptors decreases accessibility for GRK-mediated phosphorylation, this might attenuate internalization. However, we have shown previously that 5-HT_{2A} receptor internalization is independent of GRK2 and GRK5 (Gray et al., 2001), indicating a novel cell-type specific mode of regulation.

As a follow-up to elucidating the functional importance of PSD95 interaction with 5-HT_{2A} receptors, it was further demonstrated that the PDZ domain is a critical signal for the preferential dendritic targeting of this receptor in cultured cortical pyramidal neurons (Figure 1) (Xia et al., 2003b). Since disruption of the PDZ-binding domain does not result in a uniform distribution of the recombinant 5-HT_{2A} receptors, this study indicated the PDZ-binding domain of the 5-HT_{2A} receptor is essential for selective targeting of 5-HT_{2A} receptors to dendrites but not for selective exclusion from axons. The diffusion barrier at the proximal axonal segment that is composed of cytoskeletal elements such as ankyrin G and voltage gated sodium channels have been suggested to act as a neuronal sorting apparatus responsible for axonal exclusion (Sanchez-Ponce et al., 2008; Winckler and Mellman, 1999). This diffusion barrier regulates lateral movement of proteins and contributes to the maintenance of a polarized distribution of membrane proteins (Winckler et al., 1999). On the other hand, axonal exclusion of 5-HT_{2A} receptors might be due to the PDZ-motif subtype (Type 1, in this case) present in the receptor. The Type 1 PDZ-motif is essential for targeting 5-HT_{2A} receptors to apical dendrites but may not be important for axonal exclusion. Considering all the experimental evidence obtained so far, it is clear that PSD95 plays a pivotal role in regulating intracellular trafficking and function of 5-HT_{2A} receptors.

β-Arrestins: cell specific regulation of 5-HT_{2A}—Typically, GPCR function is mediated and modulated through two ubiquitous and conserved mechanisms: G-protein activity and β-arrestin function (DeWire et al., 2007; Gainetdinov et al., 2004). Agonist binding to the receptor stabilizes conformations that activate heterotrimeric G proteins; this activation leads to canonical second-messenger signaling. Within minutes of agonist binding, a GPCR is desensitized, involving a general mechanism of agonist-induced phosphorylation of the intracellular domains of GPCRs followed by binding of arrestins to the intracellular loops and carboxy-terminal tails of agonist-activated GPCRs thereby targeting the receptor to intracellular compartments (DeWire et al., 2007; Ferguson, 2001). However, several recent studies indicate not all GPCRs adopt this classic paradigm of receptor activation and desensitization, supporting a new model, in which GPCR signaling is regulated by proteins

that interact with the receptor within a cellular microenvironment (Urban et al., 2007; Violin and Lefkowitz, 2007) for which the 5-HT_{2A} receptor is paradigmatic (Gray and Roth, 2001).

The 5-HT_{2A} receptor couples principally with Gαq proteins, yet a number of studies have indicated that this receptor can have different signaling and trafficking profiles depending on the nature of the bound ligand and cellular context (Berg et al., 1998; Gonzalez-Maeso et al., 2007; Gray and Roth, 2001; Gray et al., 2001; Hanley and Hensler, 2002; Nichols, 2004). In a pioneering study, using competition binding studies with synthetic and recombinant peptides, we showed that the middle portion of the intracellular 3 loop of 5-HT_{2A} receptor binds with β-arrestins-1, -2, and visual-arrestin and also co-localizes with β-arrestin 1 and 2 in some, but not all, rat cortical pyramidal neurons *in vivo*, suggesting involvement of β-arrestins in the regulation of 5-HT_{2A} signaling (Gelber et al., 1999). In addition, these studies supported the concept derived from previous *in vitro* observations, which indicated that arrestins are involved in regulating the trafficking of GPCRs (Ferguson et al., 1996; Goodman et al., 1996). Interestingly, a large pool of 5-HT_{2A} receptors are located in intracellular endomembranes in neurons where they colocalize with β-arrestin-1 and β-arrestin-2, suggesting the arrestins may serve to scaffold the receptor in intracellular subdomains (Figure 1) (Gelber et al., 1999). However, the observation that arrestins are not ubiquitously co-expressed with 5-HT_{2A} receptors in all neurons suggest that either arrestins are not an obligatory protein in receptor signaling or that 5-HT_{2A} receptor regulation varies in different neuronal sub-populations. In HEK293 cells, we found that the agonist-mediated internalization of 5-HT_{2A} receptors was β-arrestin independent but dynamin dependent (Bhatnagar et al., 2001). Surprisingly, activation of 5-HT_{2A} receptors by agonists causes sorting of β-arrestin 1 and 2 to distinct intracellular vesicles, that did not colocalize with internalized 5-HT_{2A} receptors (Bhatnagar et al., 2001). Thus, these observations imply that β-arrestins are not required in 5-HT_{2A} receptor internalization and desensitization and they may have some additional cellular functions—at least in HEK293 cells. However, in a separate study, we demonstrated that desensitization of 5-HT_{2A} receptors was potentiated by the transient expression of dominant negative mutants of β-arrestin-1 and 2 in rat C6 glioma cells (Bhatnagar et al., 2001); however, 5-HT_{2A} desensitization is β-arrestin independent in HEK293 cells (Gray et al., 2001). Thus, this arrestin-insensitivity was clearly cell-type specific and implied novel mode(s) of regulation of 5-HT_{2A} receptors by arrestins. Furthermore, using a constitutively active arrestin mutant, Arr2-R169E, we showed that 5-HT_{2A} receptor can be internalized and desensitized even in absence of agonist (Figure 2) (Gray et al., 2003a). In addition, the interaction of the Arr2-R169E to 5-HT_{2A} receptors diminished signaling. The use of the constitutively active βarrestin demonstrates that arrestins can interact with 5-HT_{2A} receptors under basal conditions as suggested by our prior immunohistochemical studies in neurons (Gelber et al., 1999). In a recent study, Schmid et al elegantly demonstrated that two structurally distinct ligands, DOI and L-5-hydroxytryptophan (5-HTP), elicit different signal transduction and trafficking patterns of 5-HT_{2A} receptors in a βarrestin 2 dependent manner, *in vivo* and *in vitro* (Schmid et al., 2008). They also noted that arrestin-sensitivity was cell-type dependent in confirmation of our prior studies (Gray et al 2003a). Considering these studies collectively, we can assume that βarrestins have far more dramatic effects in terms of stabilizing 5-HT_{2A} receptor conformation and thereby modulating receptor trafficking and signaling than has previously been appreciated (Abbas and Roth, 2008).

Caveolin and 5-HT_{2A}: signaling and trafficking in lipid microdomains—In addition to localization and targeting of 5-HT_{2A} to the PSD, there is intriguing evidence that 5-HT_{2A} receptors are regulated by caveolins and lipid microdomains. Lipid rafts and caveolae are specialized membrane microdomains defined by their cholesterol- and sphingomyelin-rich nature, enrichment in glycosyl-phosphatidylinositol-anchored proteins, and their resistance to detergent extraction (Brown, 2006). Lipid rafts and caveolae selectively partition and organize proteins and lipids in membranes and these structures control various cellular functions

including exo- and endo-cytic trafficking, cholesterol homeostasis and transmembrane signal transduction. A growing body of evidence indicates that lipid rafts and caveolae regulate many GPCR signaling cascades by partitioning GPCRs, heterotrimeric G proteins and their various effectors in membrane microdomains (for reviews see (Allen et al., 2007; Patel et al., 2008)). Many metabotropic and ionotropic neurotransmitter receptors and neurotransmitter transporters are localized and enriched in lipid rafts and/or caveolae in glia and neurons; depending on the signaling system, these membrane domains can either facilitate or dampen neurotransmitter signaling (Allen et al., 2007; Bhatnagar et al., 2001; Donati et al., 2008; Kong et al., 2007). Two scaffolding proteins, flotillin and caveolin, are thought to scaffold and recruit proteins into lipid rafts or caveolae. Caveolins are multi-functional scaffolding proteins that are essential for forming caveolae and recruiting proteins into these lipid membrane invaginations (Cohen et al., 2004).

As part of our larger effort to identify novel 5-HT_{2A} interacting proteins, it was discovered that caveolin-1 forms a complex with 5-HT_{2A} receptors. Endogenous 5-HT_{2A} receptors co-immunoprecipitate with caveolin-1 in preparations of C6 glioma cells or rat brain synaptic membrane and also co-localize with 5-HT_{2A} receptors at the plasma membrane (Bhatnagar et al., 2004). This caveolin-5-HT_{2A} receptor interaction has profound consequences on 5-HT-mediated signal transduction. Disrupting the complex by stable knock-down of caveolin-1 and caveolin-2 by RNA interference abolishes 5-HT-induced calcium transience without effects on receptor number or serotonin binding affinity. In addition, overexpression of caveolin-1 increases the interaction between 5-HT_{2A} and Gα_q, suggesting caveolin promotes receptor-Gq coupling. Caveolin is known to interact with several G proteins, notably Gα_q (Oh and Schnitzer, 2001) and this complex between caveolin, 5-HT_{2A} and Gq could scaffold the receptor with G protein to enable efficient receptor-effector coupling. More recent studies indicate that caveolin-1 also complexes 5-HT_{2A} receptors with voltage-gated potassium channels (KV1.5) and disruption of caveolae impairs 5-HT-induced smooth muscle cell contraction (Cogolludo et al., 2006). Consistent with this, we and others have determined 5-HT_{2A} receptors are localized and enriched in isolated caveolin containing membrane fractions (Dreja et al., 2002). Phospholipase C β (PLC), the major downstream effector for 5-HT_{2A}, is similarly enriched in lipid raft/caveolae membranes isolated from astrocytes (Weerth et al., 2006). Therefore, caveolin interactions with 5-HT_{2A} may scaffold the receptor with Gα_q and PLC in lipid rafts or caveolae and this could facilitate 5-HT-mediated signaling through lipid microdomain organization.

In addition to acting as organizing centers for signaling molecules, both lipid rafts and caveolae can facilitate clathrin-independent endocytosis (Le Roy and Wrana, 2005; Rajendran and Simons, 2005) and thereby might modulate 5-HT_{2A} receptor trafficking and targeting (Figure 2). As hinted at previously, the trafficking of 5-HT_{2A} is complex and unusual in that both agonists and antagonists cause internalization and down regulation *in vitro* (Berry et al., 1996; Bhatnagar et al., 2001) and *in vivo* (Willins et al., 1999). However, the scaffolding machinery and mechanisms promoting 5-HT_{2A} internalization appear tissue specific and likely vary depending on the cellular milieu. For example, we have determined in HEK293 cells that both agonists and antagonists desensitize and internalize 5-HT_{2A} by a dynamin-dependent, but arrestin and GRK-independent mechanism (Bhatnagar et al., 2001; Gray et al., 2001). In contrast, in C6 glioma cells, 5-HT_{2A} desensitization and resensitization can be potentiated by dominant-negative dynamin or arrestin (Gray et al., 2001). Arrestin-dependent internalization of GPCRs is commonly attributed to endocytosis through clathrin-coated vesicles. Since 5-HT_{2A} can desensitize and internalize in some cells independently of GRK and arrestin, a mode of nonclathrin endocytosis may be responsible for the receptor trafficking. Caveolae-mediated endocytosis is also dynamin dependent, but distinct from clathrin-mediated endocytosis; rather than using clathrin, caveolins scaffold and recruit proteins into caveolae during endocytosis (Le Roy and Wrana, 2005). Since caveolin interacts and colocalizes with 5-HT_{2A} receptors at

both membrane and intracellular vesicles, it is very likely that caveolins and caveolae-mediated endocytosis may facilitate 5-HT_{2A} internalization (Bhatnagar et al., 2004). While it is still unclear if caveolins or caveolae regulate 5-HT_{2A} in neurons, this is being actively investigated.

Regulation of 5HT_{2A} by kinases: roles in desensitization and signaling attenuation

Studies investigating kinase regulation of GPCRs have emphasized phosphorylation as a mechanism for desensitization. A general theme of GPCR desensitization is the agonist-induced phosphorylation of intracellular domains of receptors by second messenger regulated kinases such as protein kinase A (PKA) or C (PKC) or by G protein-coupled receptor kinases (GRKs) (Gainetdinov et al., 2004); phosphorylation promotes arrestin binding to receptors which prevents G protein coupling (Ferguson, 2001). This paradigm of phosphorylation leading to desensitization has been most thoroughly explained for the β -adrenergic receptor; however, phosphorylation as a predominant mechanism for desensitization of serotonin receptors is still unclear (Roth, 2006). The current findings regarding kinase regulation of 5-HT_{2A} are summarized here and emphasize the novel involvement of p90 ribosomal S6 kinase 2 (RSK2), a kinase activated during mitogenic signaling.

Phosphorylation sites in 5-HT_{2A} and desensitization—To investigate the potential phosphorylation of 5-HT_{2A} and its role in desensitization, we identified 37 different serine or threonine residues as potential sites of phosphorylation in the putative intracellular domains of the receptor. To test if any of these intracellular residues were important for desensitization, a mutagenesis *tour de force* was employed. We systematically mutated every Ser or Thr residue to alanine individually, or in groups, and subsequently screened them for effects on 5-HT-induced desensitization (Gray et al., 2003b). It was determined that mutation of two residues to alanine, Ser188 in the second intracellular loop and Ser421 in the carboxy-terminal tail, significantly blocked desensitization. Also using alanine and deletion mutagenesis, all other Ser or Thr residues predicted to reside in intracellular domains were found not involved in desensitization. Interestingly, a single nucleotide polymorphism (SNP) of serine to phenylalanine has been identified at Ser421 in the 5-HT_{2A} (in SNP database, [rs1058576](#) at contig position 15983618). When this S421F mutant is expressed, it blocks desensitization similar to S421A, indicating this polymorphism may be important in desensitization (Gray et al., 2003b). Therefore, two key residues are essential for agonist-induced desensitization, Ser188 in the putative intracellular loop 2 and Ser421 in the carboxy-terminal tail. Questions still remain about which kinases actually phosphorylate these important sites as neither Ser188 nor Ser421 contain canonical phosphorylation consensus sites for PKC (discussed below) and it appears that 5-HT_{2A} desensitization is GRK2 and GRK5-independent (Gray et al., 2001).

Protein kinase C, calmodulin, and 5-HT_{2A} desensitization—For many years, it has been clear that activation of PKC, typically by treatment with phorbol esters, results in 5-HT_{2A} desensitization, although these effects may be cell type specific (Kagaya et al., 1990; Rahman et al., 1995; Roth et al., 1986). It is important to note, however, that phorbol ester-induced heterologous desensitization observed in these studies is not indicative of homologous desensitization that is induced by serotonin treatments. In mutagenesis studies, mutation of each of the intracellular consensus PKC phosphorylation sites had no effect on 5-HT-induced desensitization in HEK293 cells (Gray et al., 2003b), indicating that typical PKC members do not play a role in 5-HT-induced homologous receptor desensitization (similar results were found when mutating PKA or CamKII sites). Interestingly, direct PKC activation has been shown to desensitize 5-HT_{2A} in the absence of detectable phosphorylation of the receptor (Vouret-Craviari et al., 1995), suggesting that PKC may be acting downstream of the receptor to mediate desensitization, possibly by phosphorylating other signaling elements such as G proteins to reduce their coupling (Shi et al., 2007). PKC could also lead to 5-HT_{2A} desensitization by phosphorylating phospholipase C β (PLC) which has been demonstrated to

inhibit the enzyme and reduces signaling from other Gq-coupled GPCRs (Yue et al., 2000). In addition, the Ser and Thr mutagenesis data do not rule out the involvement of atypical PKC members or noncanonical PKC phosphorylation sites. Recent studies showed that PKC γ knockout elevates 5-HT_{2A} signaling *in vivo* as DOI-induced head twitch responses are significantly elevated by PKC γ knock-out, effects that are not due to changes in receptor number or agonist affinity (Bowers et al., 2006). Other recent studies suggest that calmodulin (CaM) also interacts with 5-HT_{2A} at consensus CaM binding motifs in intracellular loop 2 and the carboxy-terminal tail in a calcium dependent manner (Turner and Raymond, 2005). In this *in vitro* study, agonist-induced GTP γ S binding to membranes was reduced by addition of purified CaM, suggesting CaM inhibits G protein coupling to 5-HT_{2A}. In summary, typical PKC isoforms do not appear to contribute to homologous desensitization by phosphorylating 5-HT_{2A}, but instead are likely mediating their effects downstream of the receptor, possibly by phosphorylating G proteins or PLC. Atypical PKC isoforms (e.g. PCK γ) may be involved in desensitization but their mechanism is still unclear. Lastly, CaM may directly interfere with G protein coupling by binding to the receptor in a calcium dependent manner.

P90 Ribosomal S6 kinase (RSK2): a tonic brake for 5-HT_{2A} signaling—While direct PKC phosphorylation of 5-HT_{2A} has been elusive, recent findings indicate a novel pathway attenuates 5-HT_{2A} signaling by interactions with p90 ribosomal S6 kinase 2 (RSK2). RSK2 is a serine/threonine kinase activated downstream of multiple signaling pathways including growth factors, cytokines and the Ras-ERK-MAPK cascade involved in cell division and differentiation (Hubbard et al., 1998; Superti-Furga and Courtneidge, 1995). ERK1/2 kinases phosphorylate RSK2 promoting its activation (Chen et al., 1992) and a primary role of RSK2 is transcriptional regulation via the phosphorylation of a number of transcription factors leading to gene regulation.

In studies aimed at identifying novel 5-HT receptor interacting proteins, it was discovered that RSK2 interacts with and reduces signaling of 5-HT_{2A} receptors (Sheffler et al., 2006). Notably, several Gq-coupled GPCRs including Par-1, P2Y, Bradykinin-B, as well as the Gs-coupled β_2 -AR all show elevated signaling when RSK2 is knocked out, suggesting that RSK2 exerts a tonic break on GPCR signaling. We have demonstrated several basic features of the RSK2 interaction with 5-HT_{2A} receptors. Substantial RSK2 expression is detected through-out the neocortex with the strongest detection in layers V and VI and RSK2 colocalizes with 5-HT_{2A} receptors in layer V of the mouse prefrontal cortex (Sheffler et al., 2006). RSK2 interacts with the third intracellular loop of the 5-HT_{2A} receptor, and RSK2 coimmunoprecipitates with the receptor *in vitro* in C6 glioma cells, and in preparations of rat brain synaptic membranes *in vivo*. Loss of RSK2 resulted in increased serotonin efficacy as measured by phosphoinositide hydrolysis without a change in serotonin potency and also increased both basal and serotoninstimulated ERK phosphorylation. Recent work indicates RSK2 directly phosphorylates 5-HT_{2A} receptors and this occurs within the carboxy-terminus, suggesting RSK2 phosphorylation may desensitize the receptor, possibly by interfering with G protein coupling efficiency (Strachen et al, manuscript in preparation). In addition, since RSK2 is activated downstream of PKC and ERK activation, it is intriguing to speculate that PKC-induced desensitization of 5-HT_{2A} receptors could be mediated through RSK2, creating a negative feedback loop in which homologous or heterologous signals may modulate 5-HT_{2A} signaling.

Since RSK2 is activated downstream of multiple pathways, RSK2 may also provide a key link to crosstalk neurotrophin or cytokine signaling with serotonin signaling. While it is unclear if activation of mitogenic cascades promotes RSK2-mediated phosphorylation of 5-HT_{2A} receptors, it is compelling to speculate this event could enable neurotrophins to modulate serotonin signaling. Crosstalk between neurotrophins and 5-HT_{2A} receptors has been recently reported. Brain derived neurotrophic factor (BDNF) conditional knockout mice show a marked

reduction in 5-HT_{2A} mRNA and protein levels as well as profoundly decreased 5-HT-induced excitatory post-synaptic potentials (EPSPs) in layer V prefrontal cortex neurons (Rios et al., 2006), indicating that BDNF signaling during post-natal development is essential for normal 5-HT_{2A} expression and signaling *in vivo*. Curiously, acute treatment of rats with the 5-HT_{2A} agonist DOI greatly increases BDNF mRNA expression in pyramidal cortical neurons (Vaidya et al., 1997), suggesting reciprocal crosstalk between 5-HT_{2A} signaling and BDNF expression.

This observation that RSK2 modulates 5-HT_{2A} may also be relevant for neurodevelopmental disease research because null mutations in RSK2 in humans results in the X-linked neurodevelopmental disorder, Coffin-Lowry syndrome (CLS) (Trivier et al., 1996). CLS is characterized by moderate to severe mental retardation, pathognomonic craniofacial and skeletal deformities, growth retardation (Lowry et al., 1971), movement disorders (Stephenson et al., 2005), cardiovascular disorders, and a schizophrenia-like psychosis in heterozygote females (Hanauer and Young, 2002; Sivagamasundari et al., 1994). Recent availability of RSK2 knockout mice provide an animal model to probe the pathological causes of CLS. RSK2 knock-out mice exhibit poor coordination, impairment in spatial working memory, and exhibit long term spatial memory deficits consistent with what is found in CLS patients (Poirier et al., 2007). The novel observation that RSK2 knockout upregulates 5-HT_{2A} receptor signaling *in vitro* suggests serotonergic signaling may be dysregulated in Coffin-Lowry syndrome and this could be a contributing mechanism in CLS pathology. These observations could provide a rationale for therapeutic intervention using 5-HT_{2A} receptor antagonists to improve cognition in RSK2 knockout mice and possibly in humans suffering from CLS.

CONCLUSIONS

Results from these studies indicate that scaffolding proteins, as well as kinases, provide important regulatory input on 5-HT_{2A} receptor function. Many aspects of 5-HT_{2A} receptor pharmacology, targeting, trafficking and signaling are selectively regulated by multiple scaffolding proteins (PSD95, β -arrestins, caveolins) in a tissue specific manner, supporting the hypothesis that multiple signaling mechanisms involving scaffolding converge to regulate 5-HT_{2A}-mediated physiological functions. Proper subcellular targeting, agonist-induced trafficking and localization of the receptor with its effectors are essential; our results suggest an indispensable role for scaffolding proteins and kinases in normal 5-HT_{2A} trafficking and signaling. Disrupting 5-HT_{2A}-scaffold interactions can result in profound changes in serotonin signaling and imply that alterations in receptor scaffolding may dysregulate 5-HT_{2A} signaling in a variety of diseases. While insights into many of these interactions have been studied in cell-lines, the *in vivo* correlates of these findings in neurons are less clear. To address the *in vivo* importance of 5-HT_{2A} scaffolding, current efforts are using mouse knockout models (PSD95^{-/-}, β -arrestin^{-/-}, or caveolin-1^{-/-} mice) to determine if loss of these scaffolds impacts 5-HT_{2A} signaling *in vivo*. Future experiments using knock-out mouse models and knock-in mice expressing mutant receptors that do not interact with their scaffolding partners will provide important new insights. These exciting *in vivo* studies are certain to provide novel perspectives into the neuropharmacology and neurophysiology of this important member of the serotonin receptor family.

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FIGURE 1. Neuronal scaffolding mechanisms for sorting and targeting of 5-HT_{2A} receptors

Scaffolding proteins including PSD95, β-arrestins and caveolins interact with 5-HT_{2A} receptors and influence the subcellular targeting and signaling of the receptor. Subsequent to expression in neurons and sorting within the endoplasmic reticulum and golgi, 5-HT_{2A} receptors traffic within vesicles to various neuronal subdomains including endosomal membranes, microtubules and apical dendrites. 5-HT_{2A} receptors interact with microtubule associated protein 1A (MAP1A) which may scaffold the receptor to the microtubule cytoskeleton and facilitate trafficking to apical dendrites. 5-HT_{2A} receptors contain a canonical PDZ binding motif at their extreme carboxy-terminus which interacts with PSD95. The 5-HT_{2A}-PDZ-PSD95 interaction is essential for the polarized sorting and targeting of the receptor to dendrites. In addition to dendritic targeting, many 5-HT_{2A} receptors are found in intracellular endomembranes where they associate with β-arrestins, which may scaffold the receptor in intracellular compartments. Caveolins also interact with 5-HT_{2A} receptors which may target the receptor to cholesterol-enriched membrane microdomains of the plasma membrane to enable efficient receptor-mediated signaling. The kinase RSK2 interacts with and phosphorylates 5-HT_{2A} receptors which attenuates both basal and serotonin-induced signaling. These interactions between the receptor and scaffolding proteins or kinases provide multiple and overlapping mechanisms for sorting 5-HT_{2A} receptors and regulating serotonergic signaling.

FIGURE 2. Scaffolding proteins and serotonin-induced endocytosis of 5-HT_{2A} receptors

Agonist-induced internalization of 5-HT_{2A} receptors may involve two distinct endocytosis pathways in neurons and depend upon interactions with caveolins and/or β-arrestins. Upon serotonin binding, the receptors undergo conformational shifts that promote G protein activation and the receptors are internalized. Scaffolding interactions of the receptor with caveolins or arrestins may recruit the receptors with the appropriate endocytosis protein machinery to facilitate dynamin-dependent endocytosis through either a caveolae/caveolin or clathrin-dependent mechanism. Depending on the endocytosis pathway and scaffolding interaction, activated 5-HT_{2A} receptors traffic into caveolin containing caveosomes or clathrin-coated vesicles. The receptors are trafficked further into sorting endosomes where they may recycle back to the plasma membrane or be targeted for degradation in the lysosomal pathway. A scaffolding interaction between PSD95 and 5-HT_{2A} receptors can stabilize the receptors at the plasma membrane, possibly in post-synaptic sites, and also prevents agonist-induced internalization. Therefore, depending on the specific scaffold interaction, 5-HT_{2A} internalization can be facilitated (caveolin or β-arrestins) or prevented (PSD95).