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Review

Development of allosteric modulators of GPCRs for treatment of CNS disorders

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ABSTRACT

The discovery of allosteric modulators of G protein-coupled receptors (GPCRs) provides a promising new strategy with potential for developing novel treatments for a variety of central nervous system (CNS) disorders. Traditional drug discovery efforts targeting GPCRs have focused on developing ligands for orthosteric sites which bind endogenous ligands. Allosteric modulators target a site separate from the orthosteric site to modulate receptor function. These allosteric agents can either potentiate (positive allosteric modulator, PAM) or inhibit (negative allosteric modulator, NAM) the receptor response and often provide much greater subtype selectivity than orthosteric ligands for the same receptors. Experimental evidence has revealed more nuanced pharmacological modes of action of allosteric modulators, with some PAMs showing allosteric agonism in combination with positive allosteric modulation in response to endogenous ligand (ago-potentiators) as well as "bitopic" ligands that interact with both the allosteric and orthosteric sites. Drugs targeting the allosteric site allow for increased drug selectivity and potentially decreased adverse side effects. Promising evidence has demonstrated potential utility of a number of allosteric modulators of GPCRs in multiple CNS disorders, including neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, as well as psychiatric or neurobehavioral diseases such as anxiety, schizophrenia, and addiction.

Contents

Abbreviations: 5MPEP, 5-methyl-6-(phenylethynyl)-pyridine; 6-OHDA, 6-hydroxydopamine; 7TMR, seven transmembrane receptor; 77-LH-28-1, 1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)-quinolinone; AC-42, 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine; AChE, acetylcholinesterase; ACPT-1, (1S,3R,4S)-1-aminocyclo-pentane-1,3,4-tricarboxylic acid; AD, Alzheimer's disease; ADX71743, (+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo[d]oxazol-4(5H)-one; AFQ056, (3a*S*,5*S*,7a*R*)-methyl 5-hydroxy-5-(*m*-tolylethynyl) octahydro-1*H*-indole-1-carboxylate; APP, amyloid precursor protein; BINA, potassium 30-([(2-cyclopentyl-6-7-dimethyl-1-oxo-2,3-dihydro-1H-inden-5yl)oxy]methyl)biphenyl l-4-carboxylate; BQCA, benzylquinolone carboxylic acid; CDPPB, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; CFMMC, 3-cyclohexyl-5-fluoro-6-methyl-7-(2-morpholin-4-ylethoxy)-4H-chromen-4-one; CNS, central nervous system; CPPHA, *N*-[4-chloro-2[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl]-2-hydrobenzamide; CTEP, 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1*H*imidazol-4-yl)ethynyl)pyridine; DA, dopamine; DFB, [(3-fluorophenyl)methylene]hydrazone-3-fluorobenzaldehyde; DHPG, dihydroxyphenylglycine; ERK1/2, extracellular signal-regulated kinase 1/2; FMRP, fragile X mental retardation protein; FTIDC, 4-[1-(2-fluoropyridin-3-yl)-5-methyl-1H-1,2,3-triazol-4-yl]-N-isopropyl-N-methyl-3,6-dihydropyridine-1(2H)-carboxamide; FXS, Fragile X syndrome; GABA, γ-aminobutyric acid; JNJ16259685, (3,4-dihydro-2H-pyrano[2,3]b quinolin-7-yl)(cis-4-methoxycyclohexyl) methanone; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; L-DOPA, L-3,4-dihydroxyphenylalanine; Lu AF21934, (1*S*,2*S*)-N^1-(3,4-dichlorophenyl)cyclohexane-1,2-dicarboxamide; Lu AF32615, 4-((*E*)-styryl)-pyrimidin-2-ylamine; mGlu, metabotropic glutamate receptor; M-5MPEP, 2-(2-(3-methoxyphenyl)ethynyl)-5-methylpyridine; MMPIP, 6-(4-methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-c]pyridin-4(5H)-one; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTEP, 3[(2-methyl-1,3-thiazol-4-yl)ethylnyl]pyridine; NAM, negative allosteric modulator; NMDA, N-methyl-D-aspartate; PAM, positive allosteric modulator; PCP, phencyclidine; PD, Parkinson's disease; PD-LID, Parkinson's disease levodopa-induced dyskinesia; PET, positron emission tomography; PHCCC, *N*-phenyl-7-(hydroxylimino)cyclopropa[*b*]chromen-1a-carboxamide; PQCA, (1-(4-cyano-4-(pyridine-2-yl)piperidine-1-yl)methyl-4-oxo-4 H-quinolizine-3-carboxylic acid); SAM, silent allosteric modulator; SIB-1757, 6-methyl-2-(phenylazo)-3-pyridinol; SIB-1893, 2-methyl-6-(2-phenylethenyl)pyridine; TBPB, 1-(1′-(2-methylbenzyl)-1,4′-bipiperidin-4-yl)-1H-benzo[d]imidazol-2(3H)-one.

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Introduction

There is an urgent need for the development of new therapeutic agents for the treatment of debilitating CNS diseases, which affect millions of people worldwide. While the discovery and development of new therapeutic agents are challenging for all therapeutic areas, CNS drug discovery efforts have been especially challenging and have a very high attrition rate (Kola and Landis, 2004). The GPCRs have been among the most fruitful targets for developing drugs for the treatment of CNS disorders, as well as range of other human disease states. Many current clinical therapeutic agents act by targeting this important receptor class and downstream signaling pathways (Allen and Roth, 2011; Fang et al., 2003; Melancon et al., 2012). However, some major subfamilies of GPCRs have proven intractable in drug discovery efforts because of a difficulty in achieving high subtype selectivity and drug-like properties, including high CNS exposure, that are critical for advancing novel agents for the treatment of neurological and psychiatric disorders. Historically, drug discovery efforts targeting GPCRs have focused on the development of traditional agonists and antagonists that interact with the orthosteric neurotransmitter binding site to either mimic or block the action of the endogenous neurotransmitter or agonist. While this has been fruitful, there are many instances where the high conservation of the orthosteric binding site across related receptors prevents the development of subtype selective agents. Also, developing drug candidates based on the chemical scaffolds of the endogenous ligand may raise challenges in establishing appropriate profiles in terms of pharmacokinetic properties or brain exposure.

In recent years, advances in the development of allosteric modulators of GPCRs have emerged as promising new approaches for developing therapeutic agents that may be useful for the treatment of CNS disorders. Allosteric modulators of GPCRs bind to sites that are separate from the orthosteric binding site of the endogenous ligand and are often less highly conserved than the orthosteric site (Conn et al., 2009a). For some GPCRs, this has allowed optimization of allosteric modulators that achieve much greater subtype selectivity than is possible with traditional orthosteric ligands. In addition, allosteric modulators have other potential advantages, including ability to develop agents that have functional selectivity, allowing for potential targeting of select downstream signaling pathways, and a greater diversity of chemical scaffolds that can facilitate efforts to optimize pharmacokinetic and other drug-like properties of potential drug candidates. The surge in the development of allosteric agents has revealed a varied repertoire of drug activities, including PAMs and NAMs as well as agents with combined allosteric agonist and PAM activity and neutral ligands, termed silent allosteric modulators (SAMs) that bind to the allosteric site but do not potentiate or inhibit responses to the endogenous agonist (see Conn et al., 2009a; Melancon et al., 2012; Niswender and Conn, 2010 for reviews). In addition, allosteric agonists with a bitopic binding mode (binds to both the allosteric site and the orthosteric site) have been identified (Digby et al., 2012a; Lebon et al., 2009; Spalding et al., 2002). These varied modes of action provide tools for experimental investigation into GPCR structure and function. To date, there is a wide variety of allosteric modulators that are showing promise for potential treatment of CNS diseases (Conn et al., 2009c). Among these, some of the most advanced and well understood include allosteric modulators of the metabotropic glutamate (mGlu) receptors and the muscarinic acetylcholine receptors (mAChRs). For instance, allosteric modulators of specific subtypes of mGlu receptors have potential utility in the treatment of schizophrenia, autistic spectrum disorders, and Parkinson's disease (Morin et al., 2013b). Positive allosteric modulators of the M_1 and M_4 muscarinic receptors show promising applications in both Alzheimer's disease and schizophrenia. This is an exciting time in CNS drug discovery with several allosteric modulator candidates moving from preclinical models into clinical development.

Allosteric modulators of GPCRs

GPCRs, also called seven transmembrane spanning receptors (7TMRs), represent the largest family of cell surface receptors and are the targets of intense drug discovery efforts. While a number of available drugs on the market target GPCR signaling pathways, overall less than 20% of GPCRs are targeted (Allen and Roth, 2011). Ubiquitous receptors, these seven transmembrane-spanning proteins transduce extracellular signals for ligands as diverse as ions, photons of light, odorants and peptides into intracellular signaling cascades. Over 800 human GPCRs have been identified to date with five major families (and multiple subfamilies) based on their amino acid sequences (Katritch et al., 2013). Despite intense drug discovery and development efforts, clinically useful drugs do not exist for the large majority of these receptors. As noted above, the orthosteric binding site within GPCR subfamilies is often highly conserved, making the development of subtype specific ligands difficult. Of the orthosteric ligands developed, many of those with the highest subtype selectivity are antagonists.

Discovery and optimization of novel highly selective allosteric modulators of GPCRs have opened exciting new opportunities for the development of highly selective drug candidates for specific GPCR subtypes that were intractable using traditional approaches. While the major advances in the discovery of allosteric modulators of GPCRs have only occurred over the past decade, the principle of targeting allosteric sites on neurotransmitter receptors that act as ligand-gated ion channels has a long history and has been highly successful in developing agents for the treatment of CNS disorders (Melancon et al., 2012). The classic example of an allosteric modulator is the benzodiazepine class, which are positive allosteric modulators at the $GABA_A$ receptors (Mohler et al., 2002). These agents provide effective treatment of anxiety, sleep, and seizure disorders without inducing the adverse side effects

that would be observed with direct-acting $GABA_A$ receptor agonists. Allosteric modulators have been discovered for GPCRs, enzymes, and other ion channels (Bogoyevitch and Fairlie, 2007; Burford et al., 2013; Conn et al., 2009a; Hogg et al., 2005; Kenakin and Miller, 2010; Lewis et al., 2008; Scott et al., 2009).

As for the ligand-gated ion channels, GPCRs occupied by allosteric ligands can be modulated in a positive or negative manner (Conn et al., 2009a). The interaction of the receptor with an allosteric modulator can result in multiple pharmacological effects: *affinity modulation*, impacting the association–dissociation rate of the orthosteric ligand; *efficacy modulation*, affecting orthosteric ligand-induced downstream signaling responses; and *agonism/inverse agonism*, affecting receptor signaling in a positive or negative manner either in the presence or absence of orthosteric ligand. Ternary complex models that use cooperativity to quantify the allosteric effects are useful conceptually, but have been difficult to fit to experimental data. Using the operational model of allosterism, it is possible to quantify allosteric drug properties for use in drug development efforts. The advantages of allosteric modulators include: receptor selectivity with subtype selectivity and selective cooperativity at a given subtype as well as receptor activity dependence which maintains spatial and temporal activity dependence of endogenous signaling for those ligands which show efficacy only in the presence of the endogenous orthosteric ligand (Conn et al., 2009a; Lewis et al., 2008).

Allosteric agents have the potential to show differential effects on downstream signaling pathways, termed functional selectivity (biased agonism, stimulus trafficking). For example, $mGlu_5$ activates both intracellular calcium mobilization and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in rat cortical astrocytes. An early example of functional selectivity of allosteric modulators of GPCRs came with studies comparing the effects of the $mGlu_5$ PAMs DFB and CPPHA in native rat cortical astrocytes. While both showed similar positive modulatory effects on DHPG-induced intracellular calcium transients, their effects on ERK1/2 signaling differed (Zhang et al., 2005). There are now multiple examples in which allosteric modulators have been identified that have differential effects on coupling of the GPCR to different signaling pathways (Digby et al., 2012a; Kenakin, 2010; Maj et al., 2003; Mathiesen et al., 2005; Niswender et al., 2010; Noetzel et al., 2013; Sachpatzidis et al., 2003; Sheffler and Conn, 2008; Wei et al., 2003). Leveraging the functional selectivity of allosteric modulators of GPCRs provides a potential opportunity to develop agents that selectively target GPCR signaling pathways critical for therapeutic efficacy without modulating signaling pathways that lead to adverse effects.

Metabotropic glutamate receptors

Metabotropic glutamate (mGlu) receptors represent promising drug targets for a variety of psychiatric and neurodegenerative CNS disorders. Glutamate, the major excitatory neurotransmitter in the CNS, signals through both ionotropic and metabotropic glutamate receptors. Metabotropic glutamate receptors modulate cell excitability and synaptic transmission, as opposed to eliciting fast synaptic responses, which are mediated by ionotropic glutamate receptors. The metabotropic glutamate (mGlu) receptor family, which couple to intracellular second messengers through heterotrimeric G-proteins, includes eight members that serve neuromodulatory roles within the CNS. Members of family 3 (or Class C) GPCRs, mGlu receptors are characterized by a large extracellular "venus flytrap" N-terminal region, which serves as the glutamate (orthosteric) binding site. mGlu receptors are divided into three subgroups according to agonist binding, signaling transduction pathways, and sequence homology. Group I, which includes $mGlu_1$ and $mGlu_5$ receptors, are coupled to $G_{q/11}$ and mediate IP_3/Ca^{2+} signal transduction (Abe et al., 1992). Group II ($mGlu_{2,3}$) and Group III ($mGlu_{4,6,7,8}$) negatively couple to adenylyl cyclase and other effector systems through $G_{i/o}$ proteins. While there is abundant sequence homology within receptor subgroups at the orthosteric binding site, allosteric ligands bind to a unique topographically distinct site within the transmembrane domain. The allosteric site contains a higher level of sequence diversity between receptor subtypes as compared to the orthosteric site, allowing for greater subtype selectivity of allosteric ligands (Christopoulos and Kenakin, 2002; Conn et al., 2009a). mGlu receptors are expressed in neurons and glial cells, including astrocytes, oligodendrocytes, and microglia. In neurons, $mGlu_1$ and $mGlu_5$ are expressed postsynaptically, modulating cell excitability and post-synaptic efficacy whereas $mGlu_{2,3,4,7,8,}$ are predominately expressed presynaptically, where they can regulate neurotransmitter release (Conn and Pin, 1997). The CNS therapeutic targets for mGlus that have received the most attention include Parkinson's disease, Fragile X syndrome/autism spectrum disorders, schizophrenia, cognition, addiction, depression, anxiety and pain.

Parkinson's disease

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by motor symptoms of rigidity, bradykinesia, tremor, postural instability and gait disturbance (Jankovic, 2008). Associated non-motor symptoms include cognitive decline, mood and sleep disturbance (Chaudhuri et al., 2006). The incidence of PD in patient's older than 55 years is approximately 1% worldwide, creating a substantial disease burden in the aging world population. The pathology observed in PD patients includes progressive degeneration of dopamine neurons in the substantia nigra pars compacta (SNc) with resulting dysfunction of the basal ganglia-thalamocortical motor circuit (Fig. 1) (Dickerson and Conn, 2012; Johnson et al., 2009; Wichmann and DeLong, 1996; Zhang et al., 2005). Currently available pharmacological treatments for PD are aimed at dopamine replacement using L-DOPA or dopamine receptor agonists. While initially effective, dopamine-replacement strategies have undesired side effects including dyskinesia and have decreased efficacy over time as the disease progresses (Prashanth et al., 2011). The basal ganglia deep brain nuclei are central to the control of motor function, and the group III mGlus, including $mGlu_4$ (Bradley et al., 1999), are expressed in neurons of different basal ganglia nuclei. Dopamine (DA) depletion associated with PD in the nigrostriatal pathway leads to hyperactivity of inhibitory projections from the striatum to the globus pallidus, the first synapse in the basal ganglia "indirect pathway" (Hirsch et al., 2000). This glutamatergic overactivity of the indirect pathway is thought to contribute to the motor dysfunction (Blandini et al., 2000) and DA neuronal loss (Greenamyre and O'Brien, 1991; Przedborski, 2005) in PD patients. Clinical and preclinical studies in PD patients and animal models of PD suggest that decreasing the pathologic overactivity of this indirect pathway may be beneficial in reducing the motor symptoms associated with PD. The Group III mGlus ($mGlu_4$, $mGlu_7$, $mGlu_8$) are expressed presynaptically at different synapses in the basal ganglia circuit and are promising targets for PD (Conn et al., 2005). Of particular relevance, $mGlu_4$ is expressed presynaptically at the striato-pallidal synapse and reduces GABAergic transmission, which is overactive in PD patients following loss of dopamine neurons. The $mGlu_4$ PAMs are hypothesized to act by reducing activity within the indirect pathway (Johnson et al., 2009). Interestingly, the $mGlu_4$ subtype of mGlu receptor is expressed in presynaptic terminals of striato-pallidal projections and activation of $mGlu_4$ with the group III selective agonist L-AP4 decreases transmission at the striato-pallidal synapse by inhibiting neurotransmitter release from presynaptic terminals (Bogenpohl et al., 2013; Matsui and Kita, 2003; Valenti et al., 2003b, 2005). This effect was absent in $mGlu_4$ knockout mice, confirming a critical role for $mGlu_4$. Additionally, L-AP4 and other $mGlu_4$ receptor agonists reverse motor symptoms in preclinical rodent models of PD (Konieczny et al., 2007; MacInnes et al., 2004; Sibille et al., 2007; Valenti et al., 2003b).

The discovery of the $mGlu_4$-selective PAMs, including PHCCC (Maj et al., 2003; Marino et al., 2003) and multiple more highly optimized agents (Bennouar et al., 2013; Celanire and Campo, 2012; East et al., 2010; Jimenez et al., 2012; Jones et al., 2011, 2012a; Niswender et al.,

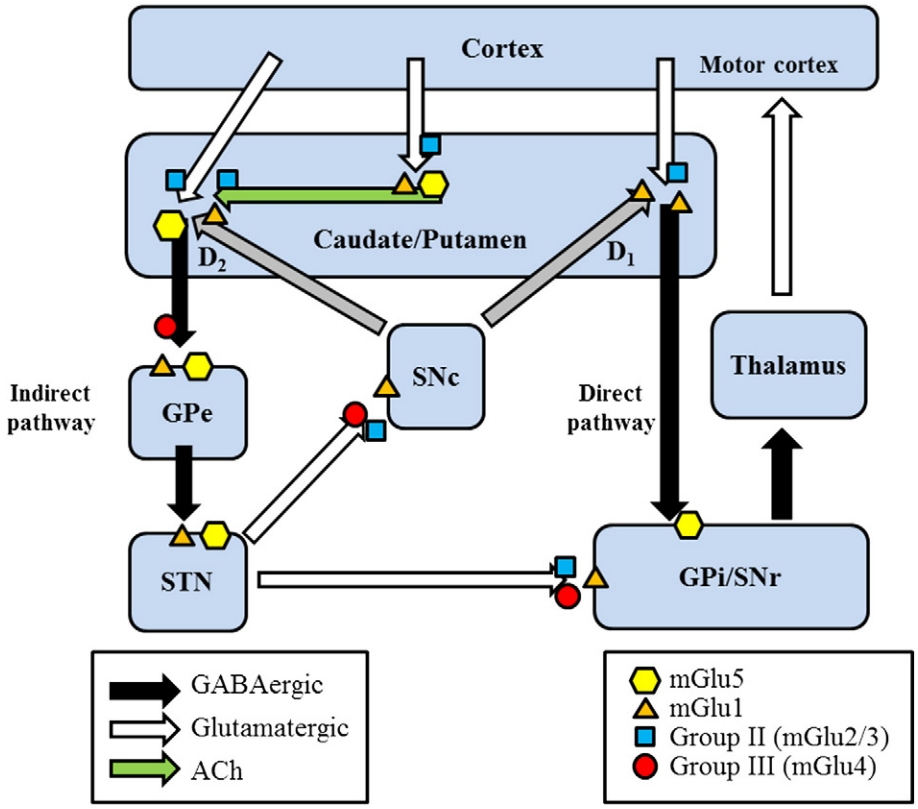

Fig. 1. Basal ganglia circuit. The basal ganglia are a collection of interconnected deep gray subcortical nuclei that are essential in the control of motor function. The major input nucleus of the basal ganglia is the striatum (caudate and putamen). The cerebral cortex sends stimulatory inputs to the striatum, including primary motor cortex. The major output nuclei include the substantia nigra pars reticulata (SNr) and the internal globus pallidus (GPi). Two major parallel pathways project from the striatum to the major output nuclei of the basal ganglia. The direct pathway contains inhibitory projections from the striatum to the output nuclei. GABAergic neurons in the striatum that express D_1 dopamine receptors project to the SNr and GPi. The indirect pathway is polysynaptic, and ends in excitatory projections to the output nuclei from the subthalamic nucleus (STN). GABAergic neurons in the striatum that express D_2 dopamine receptors project to the external globus pallidus (GPe), and subsequently the GPe sends inhibitory projections to the STN. The result of disruption of the indirect pathway is disinhibition of the STN, and net increased excitatory stimulation to the output nuclei. The balanced activity between the direct pathway inhibition of the output nuclei and the indirect pathway excitation is essential for the normal control of motor activity. The action of dopamine in the striatum decreases transmission through the indirect pathway and increases transmission through the direct pathway. Loss of dopamine neurons in the substantia nigra pars compacta (SNc) in PD leads to decreased inhibitory tone in the direct pathway with subsequent increased stimulatory tone in the indirect pathway, increased GABAergic tone at the thalamus and reduced excitation of the motor cortex. Metabotropic glutamate receptors are expressed throughout the basal ganglia. The Group I receptors ($mGlu_1$ and $mGlu_5$) are expressed in multiple locations in the basal ganglia. They are located post-synaptically, and inhibit the basal ganglia response to dopamine. Group II receptors ($mGlu_{2/3}$) are located presynaptically to the cortico-striatal, STN-SNr, and STN-SNc synapses. Of the Group III receptors, $mGlu_4$ is known to modulate corticostriatal, STN-SNr, as well as intrastriatal GABAergic synapses. Cholinergic systems modulate basal ganglia function. Of primary importance, tonically active cholinergic interneurons in the striatum release acetylcholine to modulate basal ganglia and motor function. They act project to neighboring medium spiny neurons in the striatum. Muscarinic acetylcholine receptors are expressed in the basal ganglia. M_1, M_2, M_4 receptors are expressed in the striatum, with M_5 receptors expressed on dopaminergic neurons in the ventral tegmental area (VTA) and SNc. M_4 receptors colocalize with D_1 expressing neurons in the striatum. The development of subtype specific allosteric modulators of muscarinic ACh receptors will allow for further characterization of their location and function in the basal ganglia circuit. Adapted from Dickerson and Conn, 2012; Johnson et al., 2009; Conn et al., 2005, and Wichmann and DeLong, 1996.

2008) has provided new tools that have allowed systematic studies aimed at testing the hypothesis that $mGlu_4$ PAMs may have utility in reducing motor symptoms in PD patients (Dickerson and Conn, 2012; Engers et al., 2009; Hopkins et al., 2009; Konieczny et al., 2007). Interestingly, multiple structurally diverse $mGlu_4$-selective PAMs have robust efficacy in reducing motor disability in a range of rodent models, including haloperidol-induced catalepsy and both unilateral and bilateral lesions of DA neurons using 6-OHDA (Bennouar et al., 2013; Engers et al., 2010; Jones et al., 2012a; Le Poul et al., 2012; Lopez et al., 2007; MacInnes et al., 2004; Marino et al., 2003; Taylor et al., 2003). Furthermore, the systemically active $mGlu_4$ PAMs VU0364770 (Jones et al., 2012a) and ADX88178 (Celanire and Campo, 2012) potentiate the antiparkinsonian activity of L-DOPA, showing potential as L-DOPA sparing agents.

In addition to providing symptomatic relief in PD patients, early data suggest that $mGlu_4$ PAMs may also show disease modifying effects by decreasing the degeneration of substantia nigra neurons. Thus, the $mGlu_4$ PAM PHCCC reduces DA cell death in MPTP-treated WT mice, while showing no protective effect in $mGlu_4$ knockout mice (Battaglia et al., 2006). While the precise mechanism of this neuroprotective effect is not clear, it could be mediated by a combination of reduced excitation of DA neurons (Valenti et al., 2003a), anti-inflammatory effects of $mGlu_4$ activation (Fallarino et al., 2010; Taylor et al., 2003), and other potential neuroprotective actions on DA neurons (Battaglia et al., 2006; Bruno et al., 2000). Additionally, the $mGlu_4$ PAM PHCCC as well as the Group III agonist ACPT-1 show a decrease in rodent models of neuropathic pain (Goudet et al., 2008), which may be effective for the non-motor symptoms of PD. Thus, $mGlu_4$ PAMs have potential for both symptomatic and disease-modifying treatment for PD patients. While $mGlu_4$ PAMs have not advanced to clinical testing, multiple pharmaceutical companies and academic/industry partnerships are making exciting progress that may allow direct testing of this hypothesis in clinical studies (Robichaud et al., 2011).

PD patients chronically treated with L-DOPA require increasing doses to maintain efficacy and progressively develop L-DOPA-induced dyskinesia (LID) (Fabbrini et al., 2007; Jenner, 2008; Marsden and Parkes, 1977; Meissner et al., 2011). Amantadine, an NMDAR antagonist, is currently the only drug to show clinical efficacy in LID, and the

adverse effects include cognitive impairment. Although the exact mechanism resulting in LID is not established, with decreased dopamine in the basal ganglia in PD, $mGlu_5$ is hypothesized to be involved in compensatory mechanisms and L-DOPA-induced motor complications. $mGlu_5$ is expressed in the striatum and basal ganglia (Shigemoto et al., 1993), and basal ganglia levels are increased in 6-OHDA lesioned rats (Pellegrino et al., 2007), as well as in parkinsonian primates with LID (Ouattara et al., 2010; Samadi et al., 2008). Preclinical rodent models of PD show efficacy with $mGlu_5$ NAMs to decrease LID (Dekundy et al., 2006; Levandis et al., 2008; Mela et al., 2007; Rylander et al., 2009). Fenobam, an $mGlu_5$ NAM, also decreased LID in both rodent and primate models of PD (Rylander et al., 2010). The addition of MPEP to L-DOPA treatment in parkinsonian MPTP lesioned non-human primates substantially decreased LID both in dyskinetic (Morin et al., 2010) and de novo lesioned animals (Morin et al., 2013a). Additionally, basal ganglia [^{3}H]ABP688 specific binding ($mGlu_5$) was significantly less in primates treated with MPEP combined with L-DOPA compared to L-DOPA treated animals (Morin et al., 2013b). These studies suggest that $mGlu_5$ NAMs may be useful as adjunct treatments to L-DOPA for PD. Currently, the $mGlu_5$ NAMs AFQ056 (Mavoglurant) and ADX48621 (Dipraglurant) (Rylander et al., 2010) are in phase IIa clinical studies for the treatment of LID in PD. Other possible therapeutic targets for $mGlu_5$ NAMs in addition to FXS/autism spectrum disorders and LID, include gastroesophageal reflux disease (GERD) (Keywood et al., 2009; Zerbib et al., 2010), migraine, and anxiety/stress disorders (Swanson et al., 2005).

It is important to note that the administration of $mGlu_5$ NAMs may be associated with adverse effects. For instance, the $mGlu_5$ NAM MPEP exacerbates PCP-induced psychotomimetic and cognition impairment in animal models (Brody et al., 2004a; Campbell et al., 2004) and early clinical studies suggest the possibility that $mGlu_5$ NAMs could have psychotomimetic effects in humans (Friedmann et al., 1980; Itil et al., 1978; Pecknold et al., 1982). This may be mediated by inhibition of $mGlu_5$-induced regulation of the NMDA subtype of glutamate receptor (Awad et al., 2000; Doherty et al., 2000; Henry et al., 2002; Kinney et al., 2003; Pisani et al., 2001) and the established psychotomimetic effect of manipulations that inhibit NMDA receptor function (Lahti et al., 1995; Malhotra et al., 1997). Interestingly, most $mGlu_5$ NAMs have inverse agonist activity, which may contribute to this side effect profile (Porter et al., 2005). However, recent studies have shown that it is possible to develop $mGlu_5$ NAMs with weak negative cooperativity that only partially block glutamate activation of $mGlu_5$ with full occupancy of the receptor (Rodriguez et al., 2005). While in vivo studies with these partial allosteric antagonists have not been performed, it is possible that these agents could provide clinical efficacy while minimizing adverse effects associated with full blockade or inverse agonist activity at $mGlu_5$.

Of interest, A_{2A} adenosine receptors are also expressed in the striatopallidal neurons and form oligomers with the D_2 dopamine receptor. A_{2A} receptor antagonists are pro-dopaminergic, and therefore have the potential to reduce the symptoms associated with dopamine depletion in PD (Kulisevsky and Poyurovsky, 2012). The A_{2A} receptor antagonist preladenant (SCH412384) delays haloperidol-induced extrapyramidal symptom onset in non-human primates (Varty et al., 2008). Therefore, the development of A_{2A} NAMs would provide a valuable tool for the study of dyskinesia associated with PD and movement disorders.

In addition to $mGlu_4$ PAMs and $mGlu_5$ NAMs, the development of $mGlu_2$ and $mGlu_8$ PAMs may be useful for Parkinson's disease therapy. The Group II mGlus are located presynaptically on glutamatergic axon terminals in the substantia nigra pars reticulata (SNr), potentially modulating excitatory neurotransmission (Bradley et al., 2000). Administration of group II agonists, by either the intracerbroventicular or the intranigral route, results in a reversal of akinesia in reserpine-treated rats (Dawson et al., 2000; Murray et al., 2002). Treatment of rat midbrain slices with the selective agonist LY379268 leads to long-term depression (LTD) of excitatory postsynaptic current (EPSC) amplitude in GABAergic SNr neurons. This effect was absent in $mGlu_2$ but not $mGlu_3$ knockout mice, indicating that activation of $mGlu_2$ is essential for induction of LTD in the SNr, with possible application of $mGlu_2$ agonism for the treatment of the motor symptoms of PD (Johnson et al., 2011). Non-selective group III agonists are effective in preclinical PD models. The $mGlu_8$ agonist DCPG (Thomas et al., 2001), administered by intracerebroventricular route, showed robust reversal of prolonged, but not acute, haloperidol-induced catalepsy and reserpine-induced akinesia (Johnson et al., 2013). Further, DCPG administration decreased forelimb use asymmetry in unilateral 6-OHDA lesioned rats. This evidence supports a role for $mGlu_8$ agonism in potential PD treatment. Therefore, the development of $mGlu_2$ and $mGlu_8$ PAMs may provide therapeutic benefit in PD.

Fragile X syndrome and autism spectrum disorders

Fragile X syndrome (FXS) is an X-linked monogenic disorder, and is the most common form of human inherited intellectual disability and inherited cause of autism (Santoro et al., 2012). The brains of patient's with FXS appear normal on gross examination, yet microscopically the dendritic spines demonstrate an elongate immature phenotype. Patients with FXS have poor motor coordination, tactile hypersensitivity, loose bowel movements, and an increased incidence of epilepsy. These individuals have mental disability that includes attention deficit hyperactivity, obsessive–compulsive behaviors and labile mood. FXS is most often caused by a trinucleotide repeat expansion (CGG) in 5′ untranslated region of the Fragile X mental retardation 1 (*FMR1*) gene, leading to hypermethylation and vastly decreasing or silencing the expression of the fragile X mental retardation protein (FMRP). FMRP represses translation of specific mRNAs (Bear et al., 2004) and is located in the postsynaptic region of glutamatergic synapses. Interestingly, FMRP inhibits translation of key proteins in the CNS that are stimulated by $mGlu_1$ and $mGlu_5$ (Bhakar et al., 2012). FMRP plays a critical role in long-term depression and other forms of synaptic plasticity. The absence of FMRP expression results in increased constitutive $mGlu_5$ signaling and subsequent "excessive" $mGlu_5$-mediated protein synthesis in post-synaptic dendrites with resulting dysregulation of synaptic function. Generation of *Fmr1* knockout mice with decreased $mGlu_5$ expression (50%) showed improvement in many rodent phenotypes associated with Fragile X, including rescue of neuronal spine density, supporting increased activity of $mGlu_5$ as a key component in disease development (Dolen et al., 2007). The mammalian target of rapamycin (mTOR) pathway and the ERK pathway are implicated in the coupling of $mGlu_5$ to the translational complex (Bhakar et al., 2012). The $mGlu_5$ NAMs MPEP and fenobam improve fragile X phenotypes in animal models of FXS. Furthermore, chronic pharmacological $mGlu_5$ inhibition with CTEP in the *Fmr1* knockout mouse corrected many features of fragile X in adult mice (Michalon et al., 2012). These findings highlight the importance of $mGlu_5$ in FXS, and raise the possibility that constitutive activity of $mGlu_5$ may be important in FXS. Therefore, the inverse agonist activity of $mGlu_5$ NAMs, such as is observed for MPEP, may be important for $mGlu_5$ NAM efficacy in this disease. Further studies comparing compounds with and without inverse agonist activity will determine the importance of $mGlu_5$ constitutive activity in FXS. These findings show promise for chronic $mGlu_5$ NAM treatment of patients with the FXS phenotype. A number of $mGlu_5$ NAMs are now being investigated in clinical studies for efficacy in treatment of FXS as well as other indications (Bhakar et al., 2012; Emmitte, 2013). In a small clinical trial of 30 fragile X patients, the $mGlu_5$ NAM AFQ056 (Novartis) showed improvement in patients with full methylation of the *FMR1* promoter region, demonstrating that epigenetic modification of the promoter may determine responsiveness to $mGlu_5$ NAMs (Jacquemont et al., 2011; van Bon et al., 2011). Using lymphoblastoid cell lines from FXS patients, treatment with AFQ056 did not induce demethylation of the

FMR1 gene promoter and levels of *FMR1* mRNA remained constant (Tabolacci et al., 2012).

In addition to potential utility in the treatment of FXS, recent studies raise the possibility that $mGlu_5$ NAMs may also be useful for the treatment of a broader range of autistic spectrum disorders, including idiopathic autism (Silverman et al., 2012). However, preclinical studies in mice bearing mutations that lead to tuberous sclerosis, another developmental autistic spectrum disorder, suggest that $mGlu_5$ NAMs could exacerbate symptoms and that $mGlu_5$ PAMs could have therapeutic effects. Interestingly, tuberous sclerosis complex (TSC) patients often have associated symptoms similar to Fragile X patients, including epilepsy, autism spectrum disorders, and mental disability (Tsai and Sahin, 2011). TSC mice treated with an $mGlu_5$ PAM showed reversal of cognitive defects, supporting a potential role for $mGlu_5$ in TSC (Auerbach et al., 2011). Thus, it will be critical to carefully consider specific patient populations and to develop a more complete understanding of the potential impact of $mGlu_5$ modulators in different childhood developmental disorders. FXS and TSC serve as valuable models to understand the neurobiology behind genetically complex developmental brain disorders and the potential impact of different genotypes on therapeutic response to $mGlu_5$ modulators (Krueger and Bear, 2011).

In addition to potential utility of $mGlu_5$ modulators, it is important to note that the early studies suggest that the signaling by both $mGlu_5$ and the closely related $mGlu_1$, are equally impacted by mutations that lead to FXS (Bhakar et al., 2012). Thus, $mGlu_1$ NAMs could also provide efficacy in FXS patients. While $mGlu_1$ has received less attention than $mGlu_5$ as a potential target for the treatment of FXS, Thomas et al. (Thomas et al., 2012) recently reported that selective $mGlu_1$ NAMs have robust efficacy in reversing multiple symptoms in FXS mice. Thus, it will be important to understand the potential for selective NAMs for both subtypes in treatment of FXS and related disorders.

Schizophrenia and anxiety disorders

Schizophrenia is a debilitating psychiatric disorder affecting approximately 1% of the population across the globe. The manifestation of this disorder includes a triad of symptom clusters: positive symptoms, negative symptoms, and cognitive impairment (Kim et al., 2009). Current therapies for the treatment of psychosis (positive symptoms) in schizophrenia patients focus on blockade of the D_2 dopamine receptors, and are severely limited by poor efficacy as well as adverse side effects (extrapyramidal motor symptoms and metabolic syndrome and sexual dysfunction). These combined effects limit the successful therapeutic window for the D_2 antagonists, with patients frequently switching drugs to maintain effective treatment of symptoms. Additionally, some D_2 antagonists only show partial efficacy in some patients. The finding that NMDA receptor channel blockers, including PCP, ketamine, and dizocilpine (MK-801), induce psychosis in human volunteers, led to the hypoglutamatergic theory of schizophrenia (Javitt, 1987). In addition, the administration of NMDA receptor antagonists to schizophrenic patients exacerbates both cognitive and psychotic symptoms (Lahti et al., 1995; Malhotra et al., 1997). Clinical trials using glycine co-agonists, which enhance NMDA receptor function, in combination with standard antipsychotic therapy show efficacy in decreasing negative symptoms and increasing cognition in schizophrenic patients (Coyle and Tsai, 2004).

There is a large body of evidence that Group II ($mGlu_2$ and $mGlu_3$) agonists or $mGlu_2$ PAMs provide effective action against the positive symptoms, while $mGlu_5$ PAMs may have efficacy in reducing all symptoms clusters schizophrenia patients (Herman et al., 2012). The $mGlu_{2/3}$ agonists LY354740 and LY379268 have robust efficacy in multiple rodent models of antipsychotic-like activity (Chaki et al., 2013) for both schizophrenia (Conn et al., 2008; Schoepp and Marek, 2002) and anxiolytic disorders (Schoepp et al., 2003; Swanson et al., 2005). The antipsychotic-like activity is likely to be mediated, at least in part by reduced glutamate release from presynaptic terminals on projections from the thalamus to the prefrontal cortex in rodents (Cartmell et al., 1999; Marek, 2010; Moghaddam, 2004). Further, an early clinical study with a selective $mGlu_{2/3}$ agonist showed promising effects in a phase II clinical trial in patients with schizophrenia (Patil et al., 2007). Unfortunately, this efficacy has not been reliably observed in subsequent clinical studies (Adams et al., 2013; Hopkins, 2013; Kinon et al., 2011). Experiments using $mGlu_2$ and $mGlu_3$ knockout mice provide strong evidence that the antipsychotic-like effects of $mGlu_{2/3}$ agonists in rodent models are mediated by the activation of $mGlu_2$ (Fell et al., 2008). However, it has not been possible to develop orthosteric agonists that are highly selective for $mGlu_2$ relative to $mGlu_3$.

Efforts to develop selective $mGlu_2$ PAMs have been highly successful and multiple studies reveal that $mGlu_2$-selective PAMs have robust efficacy in rodent models of antipsychotic activity that are similar to those observed with $mGlu_{2/3}$ agonists (Benneyworth et al., 2007; Cartmell et al., 1999; Galici et al., 2006; Lorrain et al., 2003; Moghaddam and Adams, 1998). ADX71149, a highly selective $mGlu_2$ PAM that is now in clinical development for the treatment of schizophrenia and anxious depression, met the primary objectives of safety and tolerability in phase I studies and advanced to phase IIa clinical testing. While the results of this important study have not yet been published, preliminary reports from part B of the Phase IIa study suggest that ADX71149 may have efficacy in reducing negative symptoms in a subgroup of schizophrenia patients (http://www.addextherapeutics.com/investors/press-releases/news-details/article/addex-reports-top-line-data-from-a-successful-phase-2a-clinical-study-with-adx71149-in-schizophrenia/).

Activation of $mGlu_4$ with positive allosteric modulators may also provide a promising therapeutic avenue for schizophrenia and the development of antipsychotic agents. The Group III $mGlu_4$-preferring orthosteric agonist LSP1-2111 showed dose-dependent inhibition of amphetamine-induced hyperactivity (Wieronska et al., 2012) and reversal of MK-801 induced deficits in novel object recognition in rats (Wieronska et al., 2013), indicating potential effects on both the positive and cognitive symptoms of schizophrenia. Further, recent studies with $mGlu_4$ PAMs show promising results in animal models that are used to predict antipsychotic effects (Slawinska et al., 2013a,b). For instance, the brain-penetrant $mGlu_4$ PAMs Lu AF21934 and Lu AF32615 showed dose-dependent reduction of amphetamine-induced hyperactivity and antagonism of 2,5-dimethoxy-4-iodoamphetamine (DOI)-induced head twitch tests in wild type but not $mGlu_4^{-/-}$ mice, supporting a key role for $mGlu_4$ in brain circuits involved in these behavioral models (Slawinska et al., 2013a). The mechanism of action for $mGlu_4$ in rodent models of antipsychotic activity are not known but are likely to be unrelated to the antiparkinsonian effects of $mGlu_4$ PAMs. Recent studies suggest that antipsychotic actions of mGlu4 PAMs may parallel the effects of $mGlu_{2/3}$ receptor agonists and $mGlu_2$ PAMs. For instance, activation of $mGlu_4$ reduces transmission at the same thalamo-cortical terminals in the prefrontal cortex that are modulated by activation of $mGlu_2$ (Zhang and Marek, 2007). In addition, activation of $mGlu_4$ reduces excitatory transmission in midbrain dopamine neurons (Valenti et al., 2005) and this could contribute to the antipsychotic-like effects of $mGlu_4$ agonists or PAMs.

In addition to $mGlu_2$-selective PAMs, recent efforts suggest that $mGlu_5$-selective PAMs may have potential as a novel approach for the treatment of schizophrenia. Activation of $mGlu_5$ receptors is known to enhance NMDA receptor function in multiple cell populations and has excitatory effects that may work in concert with NMDA receptors to increase activity in forebrain circuits that are thought to be important for the psychotomimetic effects of NMDA receptor antagonists (Brody et al., 2004b; Kinney et al., 2003; Lecourtier et al., 2007). Additionally, $mGlu_5$ receptors interact physically with NMDA receptors through intracellular scaffolding proteins (Niswender and Conn, 2010). As noted above, antagonists of NMDA receptors induce positive and negative symptoms, as well as deficits in cognitive function in humans that are similar to those observed in schizophrenia patients (Adler et al., 1998; Halberstadt, 1995; Krystal et al., 1994; Malhotra et al., 1996;

Newcomer et al., 1999; Parwani et al., 2005; Rowland, 2005). A large body of clinical and preclinical studies have led to the hypothesis that reduced activity of NMDA subtypes of glutamate receptors or brain circuits that are regulated by NMDA receptors may play an important role in the pathophysiology underlying schizophrenia (Conn et al., 2009c; Field et al., 2011; Nicoletti et al., 2011). Furthermore, multiple studies suggest that agents that enhance NMDA receptor signaling could provide efficacy in reducing the symptoms associated with schizophrenia (Coyle, 2006; Lindsley et al., 2006). Based on this, and the clear role of $mGlu_5$ acting in tandem with NMDA receptors to regulate transmission through glutamatergic circuits in forebrain regions, selective activators of $mGlu_5$ have been raised as a potential novel treatment strategy for schizophrenia that may have efficacy in reducing both psychotic and negative symptoms as well as providing pro-cognitive activity (Conn et al., 2009c). Consistent with this, $mGlu_5$ KO mice show disrupted prepulse inhibition (PPI), a model of sensory motor gating shown to be disrupted in schizophrenic patients (Brody et al., 2004a; Kinney et al., 2003). In addition, the $mGlu_5$ NAM MPEP potentiates PCP-induced psychotomimetic and cognition impairment in animal models (Brody et al., 2004a; Campbell et al., 2004). Based on these important findings, major efforts were launched to develop highly selective $mGlu_5$ PAMs. These efforts have yielded a large number of structurally diverse highly selective $mGlu_5$ PAMs and multiple studies have demonstrated that $mGlu_5$-selective PAMs have robust efficacy in rodent models of schizophrenia that predict efficacy in reducing both positive and negative symptoms (Conn et al., 2009c; Liu et al., 2008; Schlumberger et al., 2009, 2010). In addition, $mGlu_5$ PAMs potentiate multiple forms of synaptic plasticity that are thought to underlie specific aspects of learning and memory (Ayala et al., 2009; Liu et al., 2008; Rosenbrock et al., 2010; Stefani and Moghaddam, 2010), and improve cognitive function in multiple animal models (Darrah et al., 2008; Stefani and Moghaddam, 2010; Vardigan et al., 2010). These exciting studies suggest that $mGlu_5$ PAMs have the potential to provide a fundamental advance in schizophrenia therapy that could have efficacy in the treatment of all major symptom clusters of the disease.

While the more subtle approach to modulation of glutamatergic function using $mGlu_5$ PAMs has the potential to provide less adverse effect liability than would be observed with direct agonists of $mGlu_5$ or NMDA receptors, recent studies reveal that some $mGlu_5$ PAMs induce severe seizure activity (Rook et al., 2013) and excitotoxicity leading to cell death in the auditory cortex, hippocampus, and other forebrain regions (Parmentier-Batteur et al., in press). However, other $mGlu_5$ PAMs are well tolerated and it is clear that all $mGlu_5$ PAMs do not have the same adverse effect liability (Rook et al., 2013). This suggests that $mGlu_5$ PAMs likely differ in their effects on $mGlu_5$ signaling and understanding these differences and the mechanistic underpinnings of $mGlu_5$ PAM-mediated toxicity will be critical for fully developing $mGlu_5$ PAMs as potential therapeutic agents. One property that has been shown to play an important role in determining whether specific $mGlu_5$ PAMs will induce seizures and behavioral convulsions is the presence or absence of allosteric agonist activity (Rook et al., 2013). Of note, allosteric agonism is context dependent and can be influenced by differences in receptor expression. For example, Rook et al. (2013a,b) recently reported that the $mGlu_5$ PAM VU0424465 showed intrinsic agonist activity both in cell lines and in native systems, while a group of closely related compounds displayed agonist activity only in overexpressing cell lines and others showed no detectable allosteric agonist activity in any cell lines or native systems examined. Interestingly, VU0424465 showed adverse effects, including epileptiform activity, while the related compounds that were devoid of allosteric agonist activity in native systems did not (Rook et al., 2013). These adverse effects are dose-dependent and showed increased severity over time. These findings highlight the importance of selecting $mGlu_5$ PAMs lacking detectable intrinsic (glutamate-independent) agonist activity to avoid glutamate-independent receptor activity and the associated side effects. Also, as discussed above, $mGlu_5$ PAMs and other GPCR allosteric modulators can regulate specific aspects of $mGlu_5$ signaling without affecting others (Noetzel et al., 2013; Zhang et al., 2005). It is possible that some $mGlu_5$ PAMs have greater effects on signaling pathways that are involved in the adverse effect liability and that those that are biased towards other pathways could provide efficacy without the severe adverse effect liability. In addition to these different properties, recent studies suggest that hepatic metabolism of some $mGlu_5$ PAMs can yield metabolites that have robust activities that differ from activity of the parent compound and can contribute to the adverse effect liability (Bridges et al., 2013). In future studies, it will be critical to gain a clear understanding of the mechanisms underlying the different safety profiles of different $mGlu_5$ PAMs. Interestingly, in a developmental model of schizophrenia (neonatal PCP-induced cognition impairment), administration of $mGlu_5$ PAMs in adolescence prevented the appearance of delayed cognitive deficits in adult rats. Further, $mGlu_5$ PAM administration reversed the delay-induced impairment in adult rats, as evaluated by social novelty discrimination (Clifton et al., 2013). These findings suggest the exciting possibility of a preventative role for $mGlu_5$ PAM treatment in the development of schizophrenia, and further work will be important to evaluate these findings.

Addiction

Glutamatergic neurotransmission is hypothesized to play a key role in both establishment and maintenance of drug addiction (Nicoletti et al., 2011). Negative allosteric modulators of Group I ($mGlu_1$ and $mGlu_5$) mGlu receptors have potential as therapeutic agents for the treatment of addictive disorders (Achat-Mendes et al., 2012; Bird and Lawrence, 2009). The $mGlu_5$ receptors are highly expressed in the mesolimbic areas, regions central to the brain reward system. Mutant $mGlu_5$ null mice do not self-administer cocaine or exhibit locomotor-stimulating effects, despite normal levels of dopamine in the nucleus accumbens, supporting a role for $mGlu_5$ in addiction (Chiamulera et al., 2001). MPEP and MTEP treatment ($mGlu_5$ NAMs) show efficacy in cocaine abuse rodent and non-human primate models (Kenny et al., 2005; Kumaresan et al., 2009; Lee et al., 2005; Platt et al., 2008). Additionally, the novel $mGlu_5$ NAM VU0463841 shows activity in a rat model of cocaine addiction, with dose-dependent reduction of cocaine place preference and cocaine self-administration (Amato et al., 2013). Recently, $mGlu_1$ antagonism ($mGlu_1$ antagonist JNJ16259685) was reported to inhibit cocaine-induced conditioned place preference (CPP) through inhibition of protein synthesis in the ventral tegmental areas (VTA) (Yu et al., 2013).

In addition to Group I antagonists, experimental evidence suggests a potential role for $mGlu_2$ in addictive disorders. Cocaine reinforcement is elevated in $mGlu_2$ knockout mice, supporting the concept that $mGlu_2$ negatively regulates the drug reward system (Morishima et al., 2005). $mGlu_{2/3}$ agonism is associated with decreased nicotine and cocaine self-administration (Adewale et al., 2006; Liechti and Markou, 2007), providing opportunity for the development of $mGlu_{2/3}$ (likely $mGlu_2$-selective) PAMs for addiction.

Major depressive disorder (MDD)

Major depressive disorder (MDD) represents one of the most common forms of mental illness and is a significant financial and social burden (Chaki et al., 2013). Monoamine oxidase inhibitors and tricyclic antidepressants are the current mainstays of therapy for depression and show slow onset of action as well as poor efficacy. A subgroup of patients are resistant to therapy, termed treatment resistant depression (TRD). Ketamine, a noncompetitive (orthosteric) NMDA receptor antagonist, has shown considerable efficacy for treatment-resistant depression in a double blind placebo controlled trial (Zarate et al., 2006). Additional evidence of the role of hyperfunction of the glutamatergic

Table 1
Potential application of metabotropic (mGlu) and muscarinic (mACh) allosteric modulators in CNS diseases.

Receptor	MOA	CNS disease applications	Compounds
mGlu1	NAM	Neuropathic pain, FXS, anxiety/stress disorders, addiction	CPCCOEt (Annoura H et al., 1996), JNJ16259685 (Lavreysen et al., 2004; Thomas et al., 2012)
	PAM	N/A	Ro67-7476 (Knoflach et al., 2001), VU71(Hemstapat et al., 2006), Ro67-4853 (Wichmann et al., 2002), VU48 (Hemstapat et al., 2006)
mGlu5	NAM	Addiction, anxiety, chronic pain, depression, FXS (autism spectrum disorders), migraine, PD-LID,	MPEP (Gasparini et al., 1999), MTEP (Cosford et al., 2003), CTEP (Lindemann et al., 2011), Fenobam (Porter et al., 2005), AFQ056 (Jacquemont et al., 2011); M-5MPEP (partial NAM), Br-5MPEPy (partial NAM)(Rodriguez et al., 2005); GRN-529 (Hughes et al., 2013), VU046381 (Amato et al., 2013), RG7090 (RO4917523, antagonist, in clinical trials, FXS, http://clinicaltrials.gov/ct2/results?term=RO4917523)
	PAM	Anxiety disorders, Huntington's disease, schizophrenia, TSC	ADX47273 (Liu et al., 2008), VU0360172 (Rodriguez et al., 2010),VU29 (Ayala et al., 2009), LSN2463359 (Gastambide et al., 2013; Gilmour et al., 2013)
mGlu2/3	NAM	Depression	RO4432717 (Goeldner et al., 2013), MNI-137 (Hemstapat et al., 2007)
mGlu2-selective	PAM	Addiction, AD, anxiety disorders, depression, schizophrenia	BINA (Benneyworth et al., 2007; Galici et al., 2006), LY487379 (Johnson et al., 2003), ADX71149 (Hashimoto et al., 2013)
	NAM	Depression	MNI-167, RO4988546, RO5488608, RO4491533, RO4491533
mGlu3-selective	NAM	Depression	VU0463597/ML-289 (Sheffler et al., 2012)
mGlu4	PAM	Neuroinflammation, neuroprotection, PD, schizophrenia	PHCCC (Maj et al., 2003; Marino et al., 2003), VU0155041 (PAM/allosteric agonist) (Niswender et al., 2008), VU0364770 (Jones et al., 2012a), ADX88178 (Celanire and Campo, 2012), Lu AF21934 (Slawinska et al., 2013a,b), Lu AF32615 (East et al., 2010)
mGlu7	NAM	Anxiety, depression	MMPIP (Hikichi et al., 2010; Niswender et al., 2010) and ADX71743 (Kalinichev et al., 2013)
	Allosteric agonist	Anxiety, depression, PD	AMN082 (Ugolini et al., 2008)
mGlu8	Agonist	Parkinson's disease, anxiety	DCPG (Thomas et al., 2001)
M1	PAM	AD, addiction, movement disorders, neuropathic pain, PD, schizophrenia	Brucine (Lazareno et al., 1999), BQCA (Shirey et al., 2009), PQCA (Uslaner et al., 2013), ML-137 (Bridges et al., 2010c), ML-169/VU0405652 (Bridges et al., 2010e)
	Allosteric agonist	AD, movement disorders	AC-42 (Spalding et al., 2002), N-desmethylclozapine (Sur et al., 2003), TBPB (Jones, Brady et al., 2008), 77-LH-28-1(Langmead et al., 2008a), VU0184670, VU0357017 (Digby et al., 2012a; Lebois et al., 2010), VU0364572 (Digby et al., 2012b)
M4	M4-selective antagonist	Dystonia, PD	Tropicamide (Betz et al., 2007)
	PAM	AD, addiction, movement disorders, neuropathic pain, OCD, PD, schizophrenia	Thiochrome (Lazareno et al., 2004), VU152099 (Brady et al., 2008), VU152100 (Brady et al., 2008), LY2033928 (Chan et al., 2008), ML173 (Bridges et al., 2010d), ML293 (Sheffler et al., 2012)
M5	NAM	Addiction, anxiety disorders, schizophrenia	N/A
	PAM	Anxiety disorders, ADHA, PD, schizophrenia,	VU0238429 (Bridges et al., 2009) (Bridges et al., 2010b)

system in depression stems from the observation that glutamate levels in plasma and limbic brains areas are elevated in depressed patients (Sanacora et al., 2004). Modulation of glutamatergic transmission, particularly with $mGlu_{2/3}$ agonists (Feinberg et al., 2002; Fell et al., 2011), $mGlu_{2/3}$ antagonists (Campo et al., 2011; Chaki et al., 2004; Palucha-Poniewiera et al., 2010), and $mGlu_5$ antagonists (Belozertseva et al., 2007; Campo et al., 2011; Chaki et al., 2013; Li et al., 2006; Palucha et al., 2005; Pilc et al., 2013) shows promising effects as potential treatments for depression. Recently, the novel $mGlu_5$ NAM GRN-529 showed efficacy in multiple rodent models of depression, including those relevant to anxiety and pain, symptoms often associated with treatment resistant depression (Hughes et al., 2013). Furthermore, fenobam had efficacy in reducing anxiety in a clinical proof-of-concept study (Pecknold et al., 1982; Porter et al., 2005). Currently, the $mGlu_5$ antagonist RG7090 is in a phase II study for adjunctive therapy in patients with major depressive disorder (MDD) (www.clinicaltrials.gov).

Additional CNS therapeutic areas for mGlu allosteric modulators

The allosteric modulators of mGlu receptors have many other potential uses including pain, stress/anxiety disorders, and movement disorders (see Table 1) (Nicoletti et al., 2011). Neuropathic pain is a chronic condition that leads to allodynia and hyperalgesia (Schkeryantz et al., 2007). The $mGlu_1$ receptor is expressed in CNS regions essential to nociceptive processing as well as in afferent nociceptive nerve terminals (Martin et al., 1992). $mGlu_1$ knockout mice show decreased pain sensitivity (Schkeryantz et al., 2007) and administration of the $mGlu_1$-selective NAM JNJ16259685 (Lavreysen et al., 2004) is reported to show efficacy in a rodent neuropathic pain model (formalin hyperalgesia) (Mabire et al., 2005). Additionally, $mGlu_1$-selective antagonists showed in vivo activity in the spinal nerve ligation test (Bennett et al., 2012). Taken together, the development of $mGlu_1$-selective NAMs (Annoura H et al., 1996; Mabire et al., 2005) and efficacy in preclinical models are promising for the potential treatment of neuropathic pain. $mGlu_5$ NAMs show analgesic efficacy in preclinical models of neuropathic pain (Kumar et al., 2010; Montana et al., 2009). Agonists of $mGlu_{2/3}$ receptors also show analgesic effects in models of chronic and neuropathic pain, but show tolerance after chronic treatment (Jones et al., 2005).

In addition to $mGlu_4$, subtype-selective allosteric modulators for the other Group III mGlu receptors ($mGlu_7$ and $mGlu_8$) may also have potential for the treatment of CNS disorders. Recently, the $mGlu_7$ NAMs MMPIP (Hikichi et al., 2010) and ADX71743 (Kalinichev et al., 2013) have shown potential efficacy in animal models for the treatment of anxiety and depression, and provide excellent tool compounds to further elucidate the role of $mGlu_7$ in CNS diseases. As described above, the $mGlu_8$ agonist DCPG (Thomas et al., 2001) showed efficacy in preclinical models of PD (Johnson et al., 2013), and the development of $mGlu_8$ selective PAMs may provide novel therapeutics for PD patients. Recently, $mGlu_5$ PAMs showed neuroprotective effects in the BACHD mouse model of Huntington's disease (Doria et al., 2013). The continued development of mGlu allosteric modulators provides promising tools for the investigation of the role of individual receptor subtypes in CNS disease and the creation of novel therapeutics for an array of CNS disorders.

Muscarinic acetylcholine receptors

Allosteric modulators of muscarinic receptors show promise as potential therapies for a number of CNS disorders, including Alzheimer's disease and schizophrenia. Other target areas of benefit may include neuropathic and chronic pain, epilepsy, sleep disorders, Parkinson's disease, and movement disorders (Conn et al., 2009b). Acetylcholine signals through both muscarinic and nicotinic receptors. Muscarinic acetylcholine receptors are widely expressed in the CNS and include five subtypes (M_1–M_5) (see Langmead et al., 2008b for review). These Family A GPCRs respond to the endogenous agonist acetylcholine and are further subdivided into two groups based on signaling pathways. The M_1, M_3, and M_5 receptors signal through $G_{q/11}$, activating PLCβ leading to increased intracellular calcium. The M_2 and M_4 receptors signal through $G_{i/o}$ proteins and inhibit adenylate cyclase. Also, M_2 and M_4 signaling through $Gi_{\beta\gamma}$ subunits modulates ion channel activity. Muscarinic acetylcholine receptors (mAChRs) are expressed throughout the CNS, including targets for cholinergic interneurons in the striatum as well as targets of projections from the medial septum and hindbrain nuclei in the, midbrain, neocortex and limbic areas important for learning and memory. Both the M_1 and M_4 receptors are associated with learning, memory and cognition (Hasselmo, 2006; Hasselmo and Giocomo, 2006), and drugs specific for these subtypes may be useful in treatment of the cognitive symptoms associated with schizophrenia and Alzheimer's disease. The orthosteric acetylcholine (ACh) binding site is highly conserved, thwarting attempts to create an orthosteric drug with true subtype selectivity. Drugs developed for the orthosteric site lack subtype specificity and result in dose-dependent adverse side effects (bradycardia, sweating, salivation and gastrointestinal distress) from activation of the peripheral M_2 and M_3 mAChR subtypes (Heinrich et al., 2009).

Alzheimer's disease

Alzheimer's disease represents a major public health problem as the world population ages in the coming decades. The features of this progressive neurodegenerative disease include neuronal loss, behavioral and cognitive changes and decreased cerebral blood flow (Bartus et al., 1982; Hanyu et al., 2003). Acetylcholine receptor agonists and acetylcholinesterase (AChE) inhibitors improve the cognitive symptoms in Alzheimer disease patients (Feldman, 2002; Grossberg, 2002) and AChE inhibitors are among the few medications available for the treatment of AD. These cholinergic agents have limited efficacy and induce peripheral side effects, which limit their use (Lockhart et al., 2009). Studies have suggested that activation of muscarinic acetylcholine receptors may prove useful in the treatment of multiple symptoms in the spectrum of both Alzheimer's disease and schizophrenia (Langmead et al., 2008b). The muscarinic receptor orthosteric agonist xanomaline (M_1 and M_4 selective) has been shown to be effective in reducing the psychotic symptoms in patients with both schizophrenia and AD. A phase III placebo-controlled clinical trial showed that xanomeline robustly decreased psychotic symptoms in patients with Alzheimer's disease (Bodick et al., 1997a,b). While improved cognition was observed for those patients who completed the trial (high dose vs. placebo), the end-point analysis was not statistically significant for cognitive improvement. The main limiting side effect of xanomeline involved gastrointestinal associated symptoms (Bodick et al., 1997b). This study demonstrates that muscarinic agonists have potential in treating both the cognitive and behavioral aspects of Alzheimer's disease.

Both M_1 and M_4 allosteric agonists and PAMs would be desirable drug candidates for therapeutic development. The M_1 receptor is expressed in high levels in the CNS, particularly the cortex, hippocampus, and striatum (Levey et al., 1991, 1995). Efforts have focused on the development of M_1 specific ligands for AD, as M_1 is thought to be the critical subtype for cognition, attention, and sensory processing (Fisher, 2008; Langmead et al., 2008b; Robinson et al., 2011). Additionally, M_1 knockout mice show specific cognitive deficits (Anagnostaras et al., 2003; Miyakawa et al., 2001). For M_1 selective orthosteric agonists, the lack of true selectivity for M_1, coupled with high receptor reserve of M_2 and M_4 in native tissues, results in functional activity at multiple receptor subtypes, with unacceptable side effect profiles. For example, the orthosteric agonist AF267, thought to have subtype specificity for M_1, showed activity at both M_3 and M_5 receptors (Jones et al., 2008). Current M_1/M_4 selective muscarinic orthosteric ligands show intolerable side effects related to their lack of muscarinic receptor subtype selectivity. The development of muscarinic allosteric modulators has

opened a therapeutic window for treating CNS disorders with the potential to minimize peripheral muscarinic side effects.

Brucine, the first M_1 PAM identified, provided proof-of-concept for the development of M_1 subtype specific ligands (Lazareno et al., 1998). A functional screening approach identified novel M_1 PAMs, including VU0090157 and VU0029767, that showed pure PAM activity (Marlo et al., 2009) and could differentially regulate coupling to different signaling pathways. The systemically active M_1 PAM BQCA induces an increase in activation of the prefrontal cortex (PFC) and improves PFC-dependent cognitive function in a transgenic mouse model of PD (Shirey et al., 2009). In addition, BQCA has activity in other models that suggest possible efficacy in improving cognitive function and reducing psychotic symptoms (Ma et al., 2009). Recently, the M_1 PAM PQCA was shown to improve cognitive function in non-human primates, including improvements in the object retrieval detour task in rhesus macaques. Further, PQCA treatment increased frontal cortical cerebral blood flow at parallel drug concentrations, providing a potential translational biomarker for future studies (Uslaner et al., 2013). The continued development and optimization of M_1 PAMs, such as ML137, are providing exciting tools for investigative efforts into the role of M_1 in CNS disorders (Melancon et al., 2013; Poslusney et al., 2013).

Additionally, M_1 selective allosteric agonists have been developed, including AC-42 (Spalding et al., 2002), N-desmethylclozapine (Sur et al., 2003), TBPB (Jones et al., 2008) and 77-LH-28-1 (Langmead et al., 2008a). The M_1 allosteric agonist AC260584 shows antipsychotic activity in preclinical models (Vanover et al., 2008), but also is active at the 5-HT_{2A} serotonin receptor, D_2 dopamine receptor and α_{1A} adrenergic receptor (Heinrich et al., 2009). The recently developed selective allosteric agonists VU0357017 and VU0364572 showed robust efficacy in a rodent hippocampal dependent learning paradigm (Lebois et al., 2010). A number of M_1 allosteric agonists show bitopic binding (binds to both an allosteric site and the orthosteric site), including 77-LH-28-1 (Lebon et al., 2009), AC-42 (Spalding et al., 2002), and VU0364572 (Digby et al., 2012b). These allosteric agonists provided valuable tools for the investigation of M_1 in AD, but are limited by activity at other GPCRs and show complicated pharmacology with their bitopic mode of action. Furthermore, selectivity of these compounds is based on functional selectivity. Issues with high receptor reserve have made it difficult to maintain high selectivity while both optimizing for maximal M_1 potency and efficacy and achieving drug-like properties of highly selective M_1 allosteric agonists (Digby et al., 2012b).

In addition to acute actions on cognitive function, activation of M_1 reduces the pathological processing of amyloid precursor protein (APP) associated with Alzheimer's disease. TBPB, a systemically active M_1 allosteric agonist, shows decreased production of βAPP (Jones et al., 2008) and the M_1 agonist AF267B shifts APP towards the non-amyloidogenic pathway (Caccamo et al., 2006; Jones et al., 2008). Additionally, M_1 agonists show evidence of decreasing CSF Aβ42 levels in AD patients (Fisher, 2008; Heinrich et al., 2009). Therefore, drugs that activate AChRs may have disease modifying properties in AD as well as improve cognitive function.

Schizophrenia

The finding that the M_1/M_4 preferring orthosteric agonist xanomeline has antipsychotic-like effects in AD patients raises the question of whether M_1 and/or M_4 activation could also have efficacy in reducing psychotic symptoms in patients suffering from schizophrenia. The surprising finding of antipsychotic efficacy of xanomeline in AD patients prompted a Phase II clinical trial to evaluate antipsychotic efficacy of xanomeline in schizophrenia patients (Shekhar et al., 2008). Interestingly, xanomeline improved positive, negative, and cognitive symptoms in patients suffering from schizophrenia (Shekhar et al., 2008). This finding is especially interesting in light of previous studies showing that muscarinic receptor antagonists worsen symptoms in schizophrenic patients (Tandon et al., 1991) and produce psychotic symptoms in some individuals not suffering from schizophrenia or related disorders (Osterholm and Camoriano, 1982). Furthermore, analysis of postmortem brain samples from schizophrenic patients show decreased levels of M_1/M_4 receptor binding in key brain regions implicated in the disease, including the prefrontal cortex, superior temporal gyrus, hippocampus, and dorsal striatum (Crook et al., 2000; Dean et al., 2002; Deng and Huang, 2005; Zavitsanou et al., 2004). Taken together, these data raise the exciting possibility that M_1 and/or M_4 PAMs may also have potential utility in treatment of schizophrenia.

The M_4 receptor is expressed in numerous areas of the brain including the cortex, striatum, and hippocampus (Levey et al., 1995). M_4 colocalizes with the D_1 receptor in the striatum (Ince et al., 1997), suggesting a balance between cholinergic and dopaminergic neurotransmission. Mutant mice with knockout of M_4 in D_1 dopamine expressing cells (D1-M4-KO) do not show an antipsychotic response to xanomeline in rodent models of schizophrenia, highlighting the potential importance of M_4 receptors in schizophrenia (Dencker et al., 2011). Xanomeline treatment attenuates amphetamine-induced hyperactivity in M_1 knockout mice, and completely inhibits the effects of amphetamine in M_4 knockout mice, suggesting a greater role for M_4 in this process (Woolley et al., 2009). Development of the initial M_4 PAM thiochrome showed proof-of-concept for designing compounds with M_4 subtype selectivity (Lazareno et al., 2004). The discovery of a highly selective M_4 PAM, VU10010 (Shirey et al., 2008) and subsequent development of centrally active M_4 PAMs (VU0152099, VU0152100, and VU0448088) demonstrated reversal of amphetamine-induced hyperlocomotor activity in rats (Brady et al., 2008; Le et al., 2013). The M_4 PAM LY2033298 also showed efficacy in rodent models of antipsychotic efficacy, including conditioned avoidance responding and prepulse inhibition (Chan et al., 2008). LY2033298 potentiated the effect of oxotremorine (nonselective muscarinic agonist)-mediated inhibition of conditioned avoidance responding, indicative of antipsychotic properties (Leach et al., 2010). The effect was reduced in M_4 knockout mice, supporting a potential role for M_4 in models of schizophrenia. Interestingly, LY2033298 can have allosteric agonist activity under specific conditions as well (Chan et al., 2008; Nawaratne et al., 2010). These data provide strong support for the hypothesis that the antipsychotic effects of xanomeline are in part mediated by activation of M_4 and that highly selective M_4 PAMs may provide a novel approach to development of antipsychotic agents. In addition to potential efficacy in patients suffering from schizophrenia, M_4 PAMs could also provide efficacy in reducing the psychotic symptoms observed in patients suffering from AD and other neurodegenerative disorders.

Based on the discussion of M_1 PAM actions in models of AD above, it is possible that M_1 PAMs could also provide efficacy in improving cognitive function in schizophrenia patients. Interestingly, schizophrenic patients with an M_1 genetic polymorphism (*CHRM1*) had more correct responses with less perseverative errors in the Wisconsin Card Sorting Test (Liao et al., 2003). In total, the data generated thus far favors a prominent role of M_4 in psychotic symptoms and M_1 as a major contributor to specific domains of cognitive function. However, further studies are needed to develop a full understanding of the respective roles of M_1 and M_4 in the clinical efficacy of xanomeline and it is likely that M_1 activity also contributes to the antipsychotic efficacy and M_4 to the cognition-enhancing effects. The development of highly selective positive allosteric modulators for M_1 and M_4 receptors provides the tools needed to develop this understanding. Furthermore, M_1 and M_4 PAMs provide exciting new therapeutic opportunities to achieve subtype specificity with minimal peripheral muscarinic side effects and shows promise for the identification of novel therapeutics for both Alzheimer's disease and schizophrenia. Multiple companies are now focusing effort on discovery and development of selective M_1 PAMs for the treatment of schizophrenia and Alzheimer's disease. The Vanderbilt Center for Neuroscience Drug Discovery has now partnered with Astrazeneca to advance M_4 PAMs into clinical development (Jones et al., 2012b).

Hopefully, these and efforts by other companies will provide clinical assessment of the potential utility of M_1 and M_4 PAMs in AD and schizophrenia in the coming years.

M_1/M_4 agonists also have potential therapeutic applications in addiction and chronic neuropathic pain. The muscarinic receptors in the brain play a key role in the development of addiction (Sofuoglu and Mooney, 2009; Williams and Adinoff, 2008). M_1/M_4 muscarinic agonists mediate the attenuation of cocaine self-administration and cocaine discriminative stimulus, which is abolished in M_1/M_4 knockout mice (Thomsen et al., 2010, 2012). Therefore, M_1 and/or M_4 agonism is under investigation in the treatment of drug dependence and addiction. M_1/M_4 agonists show efficacy in chronic inflammatory and neuropathic pain, with xanomeline showing analgesic effects in a chronic inflammatory neuropathic pain rodent model (Martino et al., 2011).

M_5 modulators

The M_5 muscarinic receptor subtype comprises less than 2% of the CNS muscarinic receptors, and is expressed in both the cerebrovascular system and midbrain dopamine neurons (Weiner et al., 1990). The M_5 receptor is the sole muscarinic subtype detected in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) (Vilaro et al., 1990; Weiner et al., 1990), where it is coexpressed with D_2 dopamine receptors, suggesting a role for M_5 in the modulation of dopaminergic transmission (Weiner et al., 1990). Modulation of dopaminergic function using M_5 PAMs or NAMs has been raised as having potential utility in the treatment of addiction (Raffa, 2009) and could also have utility for the treatment of other disorders that involve changes in dopaminergic function, including schizophrenia, and attention-deficit hyperactivity disorder (ADHD). The combination of the genes for M_5 (CHRM5) and α7 nicotinic acetylcholine receptor show linkage with schizophrenia susceptibility in humans (De Luca et al., 2004). Additionally, the M_5 knockout mouse shows decreased amphetamine-induced hyperlocomotion (Wang et al., 2004). The localization of the M_5 receptor to dopaminergic midbrain neurons and association with a preclinical model of schizophrenia is encouraging for research into the role of M_5 in schizophrenia. Studies of M_5 knockout mice suggest that M_5 may be a target in AD due to cerebrovascular tone regulation (Yamada et al., 2001) as well as cognitive function. Until recently, no selective M_5 ligands existed, making it difficult to fully evaluate the potential utility of M_5 modulators in animal models related to these disorders. However, the recent discovery of highly selective M_5 PAMs (Bridges et al., 2009, 2010a) provides novel tools for the investigation of the role of M_5 in CNS disorders.

Potential utility of M_4 NAMs in treatment of Parkinson's disease and dystonia

Historically anticholinergic agents were used as treatments in PD, beginning with the deadly nightshade *Atropa belladonna*. The concept of striatal dopaminergic–cholinergic antagonism developed with findings that with decreased dopamine in the striatum there is evidence of increased acetylcholine levels in PD (Salamone et al., 2001). Anticholinergics in PD act through muscarinic receptors, and acetylcholinesterase inhibitors (such as physostigmine) are known to worsen PD symptoms (Bourke and Druckenbrod, 1998; Ott and Lannon, 1992). Drug-induced tremulous jaw movements serves as a recent model for the resting tremor associated with Parkinson's disease. Tremulous jaw movements induced by the muscarinic antagonist pilocarpine are robustly decreased in M_4 knockout mice, suggesting that the M_4 subtype plays a key role (Salamone et al., 2001). Additionally, the M_4-preferring antagonist tropicamide inhibits tremulous jaw movements in a rodent model of Parkinson's disease (Betz et al., 2007). Therefore, there is therapeutic potential for use of M_4 NAMs to control tremor associated with PD. M_4-targeted therapies have potential applications in other movement disorders, including dystonia and Huntington's disease, where there is evidence of cholinergic dysfunction (Pisani et al., 2007; Smith et al., 2006). Also, muscarinic antagonists are among the few agents that can provide efficacy in treatment of generalized dystonia (Jankovic, 2006; Martella et al., 2009). It is possible that highly selective NAMs for M_4 or another mAChR subtype could provide antidystonic efficacy without the adverse effects observed with non-selective mAChR antagonists.

Optimization of allosteric modulators as drug candidates

Development and optimization of allosteric modulators of GPCRs as drug candidates present multiple challenges (Conn et al., 2012; Klein et al., 2013; Melancon et al., 2012). The success of lead compound development for a number of allosteric modulators of GPCRs has established a set of drug optimization strategies for this class. With the nonlinear or "flat/shallow" nature of SAR for many members of this drug class, designing libraries around a central core by focusing on "islands" of constituents has led to successful lead optimization (Nawaratne et al., 2010; Wood et al., 2011). Also, it is critical to focus attention on both SAR of potency and cooperativity of allosteric modulators as well as differential effects on different aspects of receptor signaling (Wootten et al., 2013). Attention to principals emerging from medicinal chemistry optimization of allosteric modulators increases the opportunities for advancing successful drug candidates in this class. Once a hit compound is identified via high throughput screening, chemical tractability should be established through the synthesis of appropriate focused iterative compound libraries to generate lead compounds. The use of systematic fluorine substitution strategies has been successful for identifying fluorinated cores tolerant of change, and proved successful for M_1 PAM development (Bridges et al., 2010d; Reid et al., 2011; Yang et al., 2010). Therefore, fluorine substitution may prove a good first tier strategy for the optimization of hit compounds. Selecting hit compounds with the combination of both tractable SAR and encouraging physicochemical properties has proven successful (Kenakin and Miller, 2010). It is essential to fully characterize lead compounds using radioligand binding to establish in vitro receptor interaction. Running multiple (secondary/parallel) functional screens (such as GTPγS if primary screening performed was FLIPR calcium assay) will help to avoid stimulus bias in cases where biased ligands are not desired. In addition, allosteric modulators may show differential signaling depending on the cellular context used for screening (Niswender et al., 2010). The use of in vitro native systems, such as brain slice electrophysiology, supports biological relevance of lead compounds. It is also important to note that the choice of orthosteric compound is critical for allosteric modulator screening and optimization efforts, as the cooperativity between the allosteric modulator and orthosteric site can change depending on the orthosteric probe ligand, a concept termed "probe dependence" (Kenakin, 2005; May and Christopoulos, 2003). Use of the endogenous orthosteric ligand increases the likelihood of physiological relevance. Screening assays using non-native agonists should be interpreted with the caveat of probe-dependent pharmacology. Additionally, allosteric sites vary across species and may lead to differences between in vitro screens and in vivo animal model pharmacology. While cell line systems that allow relatively high throughput are critical for screening compounds in a chemistry program, it is critical to be mindful that there are instances in which there is a disconnect between effects of allosteric modulators in cell based assays and in biologically relevant native systems and in vivo assays. The differences in pharmacology observed in cell based assays as compared to native systems can stem from undetected stimulus bias, or context-dependence of allosteric modulator action and can make allosteric modulator optimization especially challenging. This can be further complicated by "molecular switches" in which subtle changes to the structure of a compound changes the mode of pharmacology of allosteric modulators (i.e. mode switching with PAM to NAM/SAM conversion with subtle structural changes) and/or changes the receptor subtype selectivity can confound lead compound development (Bhagwanth et al., 2012; Gregory et al., 2013; Melancon et al., 2012; Utley et al., 2011;

Wood et al., 2011). The screening assays used to identify lead compounds have a limited ability to detect weak or partial allosteric modulators, which may prove to be useful compounds for further development (Christopoulos and Kenakin, 2002). This is especially important when metabolites of administered allosteric modulators have pharmacological activity that is different from that of the parent compound and thereby confound interpretation of in vivo effects of systemically administered allosteric modulators (Bridges et al., 2013). It is recommended to use compounds of the same chemotype and demonstrate competitive interactions with test compounds for both ex vivo occupancy studies and in vivo imaging studies. In summary, the drug discovery effort for allosteric modulators has provided valuable insight into optimal methods for hit-to-lead development. While the complex pharmacology of mode switching, functional bias and bitopic ligand activity complicate the discovery process for allosteric ligands, they provide novel insight into the function of GPCRs. In fact, functional selectivity may become an asset for allosteric modulators targeting disease states linked to a specific signaling pathway.

Future opportunities

Allosteric modulators of GPCRs represent exciting drug candidates for the treatment of an array of CNS disorders. In addition to the metabotropic and muscarinic allosteric modulators detailed above, drugs have been developed targeting a number of other GPCRs (see Conn et al., 2009a for review and AlloSteric database (http://mdl.shsmu.edu.cn/ASD/)) (Huang et al., 2011). Several drugs have successfully entered the marketplace, providing proof of concept for allosteric modulators as clinical therapeutics. Cinacalcet (Sensipar), a PAM for the calcium sensing receptor (CaSR), is currently used to treat patients with hyperparathyroidism (Davey et al., 2012; Nemeth et al., 2004). Maraviroc (Selzentry), a NAM of chemokine receptor 5 (CCR5) (Garcia-Perez et al., 2011), prevents HIV-1 cellular entry. In addition to GPCRs, allosteric modulators have also been developed for enzymes including allosteric kinase (Akt) inhibitors (Lindsley et al., 2005) and phospholipase D (PLD) (Lavieri et al., 2010; Scott et al., 2009).

Advantages of allosteric modulators of GPCRs include subtype selectivity and functional selectivity. With the emerging literature on signaling bias, it may be possible to tailor allosteric development to target specific downstream receptor pathways, avoiding undesirable side effects elicited by parallel signaling pathways. Due to the functional selectivity possible with allosteric ligands, it is crucial to incorporate multiple functional assays into drug screening paradigms. Probing multiple pathways will allow for early detection of pathway dependent allosteric modulation (Conn et al., 2009a). The development and utilization of the operational model of allosterism allows for characterization and quantification of allosteric drug properties. In addition, it may be possible to utilize partial or silent allosteric modulators to avoid adverse effect liability. PAM ligands may also exhibit allosteric agonist activity, termed ago-potentiators, a feature that may prove advantageous in certain CNS diseases. As a part of drug development in the neurodegenerative and psychiatric area, there is increased emphasis on early demonstration of in vivo target engagement using microPET imaging in non-human primate models, with development of radioactive ligands critical to this effort (Lee and Farde, 2006; Marik et al., 2011). This PET data is especially important in the absence of specific disease biomarkers, and can help with in vivo dose selection to avoid under or overdosing. The surge in efforts to develop allosteric modulators of GPCRs and the collaboration between industry and academics paints a promising picture for the effort to develop effective treatments for CNS diseases.

Disclosures

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