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Integrated approaches to understanding antipsychotic drug action at GPCRs

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Abstract

The G-protein coupled receptor (GPCR) family of genes represents one of the largest druggable families of genes in the human genome. This is evident by the fact that approximately 30% of currently marketed drugs target GPCRs. However, many of these drugs are limited in their clinical potential as they are associated with debilitating side effects – a consequence of our incomplete understanding of their pharmacology and the signaling pathways regulated by GPCRs. Because of the limited range of tools available to resolve these issues, integrated approaches are required to fully understand the pharmacological action of drugs and the biochemical repertoire regulated by GPCRs. In this review we will focus on the action of antipsychotic drugs on certain monoamine GPCRs in the central nervous system (CNS) and the approaches being developed to elucidate their distinct pharmacology.

Introduction

The major monoamine neurotransmitters in the brain are dopamine (DA), serotonin (5-HT), and norepinephrine (NE) [1]. Monoamine dysfunctions have been implicated in various CNS disorders such as attention-deficit/hyperactivity disorder, drug abuse, Parkinson's disease (PD), schizophrenia, depression and bipolar disorder [2–6]. Antipsychotics are a major class of psychotropic drugs that are used in the treatment of schizophrenia and mood disorders. These antipsychotic drugs are GPCR ligands (agonists, antagonists, inverse agonists and partial agonists) that have been used extensively to study the effects of monoamine GPCR activation or inhibition. Several *in vitro* and *in vivo* assays to study antipsychotic action at GPCRs have been developed and we will suggest how these can be integrated with novel approaches to give us a better understanding of GPCR mechanisms of action.

The "typical" antipsychotics discovered in the 1950s, including chlorpromazine and haloperidol, are clinically effective but have serious side effects, such as extrapyramidal symptoms (EPS) and hyperprolactinemia. However another drug, clozapine, belonging to the later "atypical" antipsychotic category, has comparable clinical efficacy but no EPS [7], although it does display a suite of other adverse effects. The prevalent hypothesis prior to the

1970s was that dopamine was involved in the mechanism of action of these drugs, yet no GPCR targets were identified. Pioneering work by Seeman *et al.* and Creese *et al.* [8,9] using competition binding experiments with [^{3}H]-haloperidol and [^{3}H]-dopamine showed that the common property of all antipsychotics was their ability to bind to DA receptors in striatal homogenates, supporting the "DA hypothesis" of schizophrenia. Several years later Meltzer *et al.* [10] showed that antipsychotics could be classified as typical or atypical based on their binding affinities to DA D2 and serotonin type 2A (5-HT2A) receptors. However, antipsychotics have also been shown to bind to other GPCRs such as serotonin 5-HT1A [11], alpha-adrenergic α1 and β2, histamine H1 and muscarinic acetylcholine M1 receptors [12]. While the efficacy of most clinically effective antipsychotics can be attributed to D2 and 5-HT2A receptors, compounds with distinct pharmacology such as the specific 5-HT2A receptor inverse agonist pimavanserin may find applications in particular conditions such as PD psychosis [13]. Owing to the diverse pharmacological profile of antipsychotics, a better understanding of their action is essential. Although clinical studies in humans provide invaluable information, both cellular and animal models are necessary to elucidate the mechanisms of action of antipsychotics.

In vitro approaches to elucidate antipsychotic action at GPCRs

Historically, the classification of antipsychotics has been made by interpreting data from binding studies of DA or 5-HT2 receptors in tissue. However, the discovery of several families of G proteins [14], the cloning of GPCRs and the observation that a GPCR can activate multiple G proteins [15] have in recent years caused an explosion in the development of assays that can reveal the regulation of multiple downstream signaling cascades. Several high-throughput screening (HTS) assays have been developed such as aequorin-based fluorescent assays [16] or cyclic adenosine monophosphate (cAMP) response element-directed reporter assays [17] to measure $G\alpha_q$, $G\alpha_i$, and $G\alpha_s$ signaling events. Additionally, fluorescence resonance energy transfer assays, such as a protein kinase A (PKA) cAMP sensor [18] and an EPAC-based sensor [19], have been developed to study $G\alpha_i$ and $G\alpha_s$ signaling downstream of several GPCRs. Recent advances have been made through development of bioluminescence resonance energy transfer (BRET) [20] assays to study GPCR-dependent cAMP signaling [21]. Using a reporter-based assay to measure G protein signaling at DA and 5-HT2 receptors, Weiner *et al.* [22] have revealed that most antipsychotics are antagonists or inverse agonists at DA D2 and 5-HT2A receptors, supporting the earlier observations from binding studies. Moreover, recent findings have shown that several antipsychotics are partial agonists at the 5-HT1A receptor G_i pathway [23], and this property might provide an explanation for the better side effect profile of atypical antipsychotics [24].

Interestingly, in recent years a new paradigm in GPCR signaling has emerged, wherein GPCRs mediate their cellular functions by two distinct mechanisms, the canonical G protein-dependent signaling pathway and a G protein-independent β-arrestin-mediated form of signaling [25]. This new paradigm emerged from observations that beta-arrestin, which normally mediates desensitization [26] and internalization [27] of GPCRs, can mediate GPCR signaling through its ability to scaffold kinase complexes [28,29]. β-Arrestin-dependent signaling is both temporally and spatially separated from G protein signaling

[30,31]. The discovery of these two separate modes of signaling has given rise to the concept of biased signaling at GPCRs [32], although a similar but even broader concept termed "functional selectivity" had been proposed previously [33]. This new signaling paradigm, along with initial observations that β-arrestin2 knockout (βarr2KO) mice show reduced DA-dependent locomotion in response to psychotropic drugs such as morphine and amphetamine [34,35], has led to a reanalysis of cellular assays and screening technologies for antipsychotics. Historically, the effects of antipsychotics had been analyzed only at G protein-coupled responses; but since these discoveries, antipsychotic profiling now focuses on both G protein- and β-arrestin-dependent signaling pathways. Antipsychotics that were classified as agonists, antagonists or partial agonists now need to be reclassified based on their activity not only at the G protein pathway but also at the β-arrestin pathway. Some studies have determined the activity of antipsychotics at interactions of β-arrestin2 and the D2 DA receptor [36,37], but no similar studies of the 5-HT2A receptor-arrestin pathway have yet been published, probably due to the fact that ligands at these receptors function via both arrestin-independent and -dependent mechanisms in different cell types [38,39]. Using a BRET-based approach, Masri *et al.* [36] showed that all clinically effective antipsychotics uniformly display antagonist activity at D2 receptor-βarr2 interactions. This study correlates well with the observation that amphetamine-induced hyperlocomotion is reduced in βarr2KO mice and highlights the need to associate *in vitro* and *in vivo* observations (Table 1).

In vivo approaches to understanding antipsychotic action at GPCRs

Rodent models remain the most prevalent experimental paradigm for *in vivo* studies. Pharmacological rodent models that have been used to screen for antipsychotic efficacy are based on amphetamine- (Amph) and phencyclidine- (PCP) induced hyperactivity. Although these models do not recapitulate all the symptoms of schizophrenia observed in humans-including the positive (hallucinations, delusions and disorganization), negative (alogia, avolition and anhedonia) and cognitive symptoms - they have both reliability and predictive validity when assessing antipsychotic action [40]. These pharmacological models are analyzed in common behavioral tests corresponding to schizophrenia endophenotypes, such as inhibition of hyperlocomotion, reversing prepulse inhibition (PPI) disruption, social behavior, conditional avoidance response, and latent inhibition ;and additionally in determining the side effect profile by measuring catalepsy (as a measure of EPS), prolactin secretion, agranulocytosis and glucose levels [24,41–43]. These pharmacological models have revealed that all antipsychotics are efficacious in reversing hyperlocomotion and PPI [42], which are considered endophenotypes of positive symptoms of schizophrenia [41]. However, although some atypical, but no typical, antipsychotics are moderately efficacious in reversing endophenotypes of negative symptoms in animal models, contradictory evidence in human patient studies [44–46] suggests that reversal of negative symptoms in schizophrenia is a largely unmet need and requires further investigation. Recent evidence from human studies and certain animal models has suggested a role for prefrontal cortical DA D1 receptors in the manifestation of negative symptoms and cognitive deficits in schizophrenia [45,47]. Interestingly, most antipsychotics have weak binding to DA D1 receptors, suggesting an avenue for further research.

In addition to these pharmacological models, genetic models have also been used to screen for antipsychotics. Several genetic models, such as DA transporter knockout [48], NMDA receptor subunit 1 knockdown [49] and disrupted in schizophrenia (DISC1) knockout [50] mice, recapitulate positive and/or negative symptoms of schizophrenia, and some of these models can be used to screen for novel antipsychotics. One of the advantages of using animal models is to delete or overexpress a particular gene of interest to test its role in endophenotypes of schizophrenia. However, a genetic approach requires a valid rationale, perhaps established through association or pathology studies, to target a particular gene, since generating a mouse model can be an expensive and time-consuming endeavor. But a genetic targeting strategy leading to amelioration of schizophrenia endophenotypes could provide new targets for antipsychotic therapies. Several ligands against novel targets such as partial agonists to β-arrestin2 (βarr2) at D2 receptors [51] and agonists to neurotensin receptor NTR1 [52], metabotropic glutamate receptor mGluR2 [53] and nicotinic acetylcholine receptors [54], which could potentially lead to new antipsychotic therapies, have been identified through genetic animal models.

Several studies have used receptor knockout mice such as 5-HT2A, 5-HT1A and D2 knockout mice to study the effects of antipsychotics on schizophrenia endophenotypes [55–58]. However global receptor knockout studies do not allow facile interrogation of cell-specific effects of drugs on receptor signaling pathways or behaviors. To study the role of cell-specific signaling pathways in schizophrenia endophenotypes and antipsychotic action, several strategies have emerged over the past few years by combining genetic targeting approaches. A technique further developed by the Gene Expression Nervous System Atlas (GENSAT) project, termed bacterial artificial chromosome (BAC) transgenic technology, targets GPCR-specific neuronal populations with promoter-specific reporters that label [59,60] or Cre recombinase that deletes [61] any gene of interest when combined with a mouse line expressing a “floxed” gene. Several studies with these Cre lines have provided useful insights into the action of antipsychotics. Bateup *et al.* [62] showed that mice with a deletion of DARPP32 (dopamine and cAMP regulated phosphoprotein of 32kDa molecular weight) in either D1 or D2 receptor-expressing neurons had a reduced cataleptic response when treated with haloperidol. DARPP32, as its name implies, is a canonical downstream G-protein effector that is regulated by DA receptors through the cAMP/PKA pathway [63], although its phosphorylation is regulated in opposing fashion by D1 and D2 DA receptors upon haloperidol treatment [64]. Thus these studies suggest that haloperidol, which is an antagonist at both D2 and D1 receptors, probably causes catalepsy due to inhibition of the G-protein pathway at both receptors. A similar study by our group analyzed the effect of deletion of the other arm of the GPCR pathway, i.e. the beta-arrestin pathway in specific neuronal populations [65]. We showed that in either D1 or D2 neuron-specific knockouts of GSK3β [65], which is downstream of D2R-βarr2 signaling [35,66,67] haloperidol still caused catalepsy. These combined results suggest that antagonism of the G protein pathway and not the βarr2 pathway (through GSK3β) is predominantly responsible for the cataleptic response caused by haloperidol. Moreover, in mice with GSK3β deletion in D2 neurons we found that aripiprazole, but not haloperidol, lost its ability to antagonize Amph-induced locomotion, suggesting that aripiprazole acts predominantly through the βarr2 pathway to alleviate this endophenotype of schizophrenia. The atypical antipsychotic aripiprazole,

unlike haloperidol, is a partial agonist at the G-protein pathway (Table 1) but, similar to haloperidol, is an antagonist at D2/βarr2 interactions. These data therefore suggest that antagonizing the βarr2 pathway at the D2 receptor might alleviate psychosis without causing catalepsy. Interestingly, 5-HT1A and 2A receptor agonists have been shown to attenuate haloperidol-induced catalepsy in rodents [68] and several "atypical" antipsychotics are partial agonists at 5HT1A receptors. These results furthermore highlight the fact that functional selectivity can be observed at the behavioral level as well (Figure 1A). In a recent study Allen and colleagues [51] generated novel β-arrestin-biased ligands based on the aripiprazole scaffold and showed that these novel β-arrestin biased antipsychotic-like compounds are efficacious at inhibiting Amph- and PCP-induced hyperlocomotion without inducing catalepsy.

Transcriptomic and proteomic approaches

A concerted research effort involving both *in vitro* and *in vivo* studies can be successfully employed to identify novel signaling pathways. However, cellular assays and animal behavior models cannot be efficiently used to characterize the comprehensive molecular effects of drugs throughout the brain. Recently a translational profiling approach called Translational Ribosome Affinity Purification (TRAP) was developed to identify the molecular determinants of different cell types in the brain [69,70]. This technology uses a BAC mouse line that expresses an EGFP-tagged L10a subunit of the ribosomal machinery in a cell-specific manner, which can then be used to affinity purify L10a-bound actively translating mRNA transcripts. An alternative but similar approach involves the use of a mouse line expressing an HA-tagged L22 ribosome subunit that is Cre-inducible [71]. Such techniques provide more sensitive and cell-specific data compared to microarrays, and many more targets can be identified by using the latest whole-transcriptome sequencing technologies such as RNAseq [72,73]. Although mRNA studies have been done before [74,75], TRAP can potentially be used to assess the effects of psychotropic drugs on the mRNA profile in various cell types in the brain. One potential application would be a comparative study of cell-specific mRNA profiles induced by endogenous versus functionally selective GPCR ligands. Such studies might provide an opportunity to identify previously unacknowledged novel targets for the development of more selective therapies with fewer side effects.

Transcriptional profiles provide valuable information about the genes that are active under particular conditions but they do not necessarily translate into similar protein profiles. Several techniques have been utilized to assess protein profiles under different conditions. One of the most widely used techniques is mass spectrometric analysis of cellular or tissue samples [76]. A relevant example of mass spectrometric methods to identify proteomic profiles upon GPCR stimulation was done by Xiao *et al.* [77], where the authors aimed to identify binding partners to β-arrestins under basal or stimulated conditions at the angiotensin AT1a receptor (AT1aR). The authors found several previously known interactors of β-arrestins and in addition identified several novel partners, which they confirmed by co-immunoprecipitation experiments. Furthermore, in a separate study the same group analyzed the phosphoprotein profile when the AT1aR is activated by a β-arrestin biased ligand Sar(1), Ile(4), Ile(8)-angiotensin (SII) [78]. The success of these studies provides impetus to

perform further cell-specific protein profiling *in vivo* similar to TRAP. By combining BAC technology, cell-specific protein labeling such as biotinylation [79,80] and mass spectrometry, cell- or receptor-specific protein profiles can be determined upon exposure to various drugs. Such integrated approaches may provide insights into the actions of drugs at the translational and post-translational level.

In summary we have reviewed the various approaches used to elucidate the effects of antipsychotic drugs on GPCR signaling in the brain, including novel techniques that have gained prominence in the past several years. Integrating all or some of these methods should allow investigators to not only validate signaling pathways but also to identify non-canonical ones (Figure 1B). In addition, these integrated approaches will continue to provide valuable additional insights into the mechanisms of antipsychotic action at therapeutic and non-therapeutic targets. Such techniques are critical in the development of novel therapies that are more pathway-specific and efficacious with minimal off-target effects.

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Highlights

1. Historical perspective of antipsychotics and screening technologies
2. Current developments in antipsychotic screening methods
3. In vivo approaches to study and develop antipsychotics
4. Developments in transcriptomic and proteomic approaches
5. How different approaches can be integrated to better understand antipsychotic action

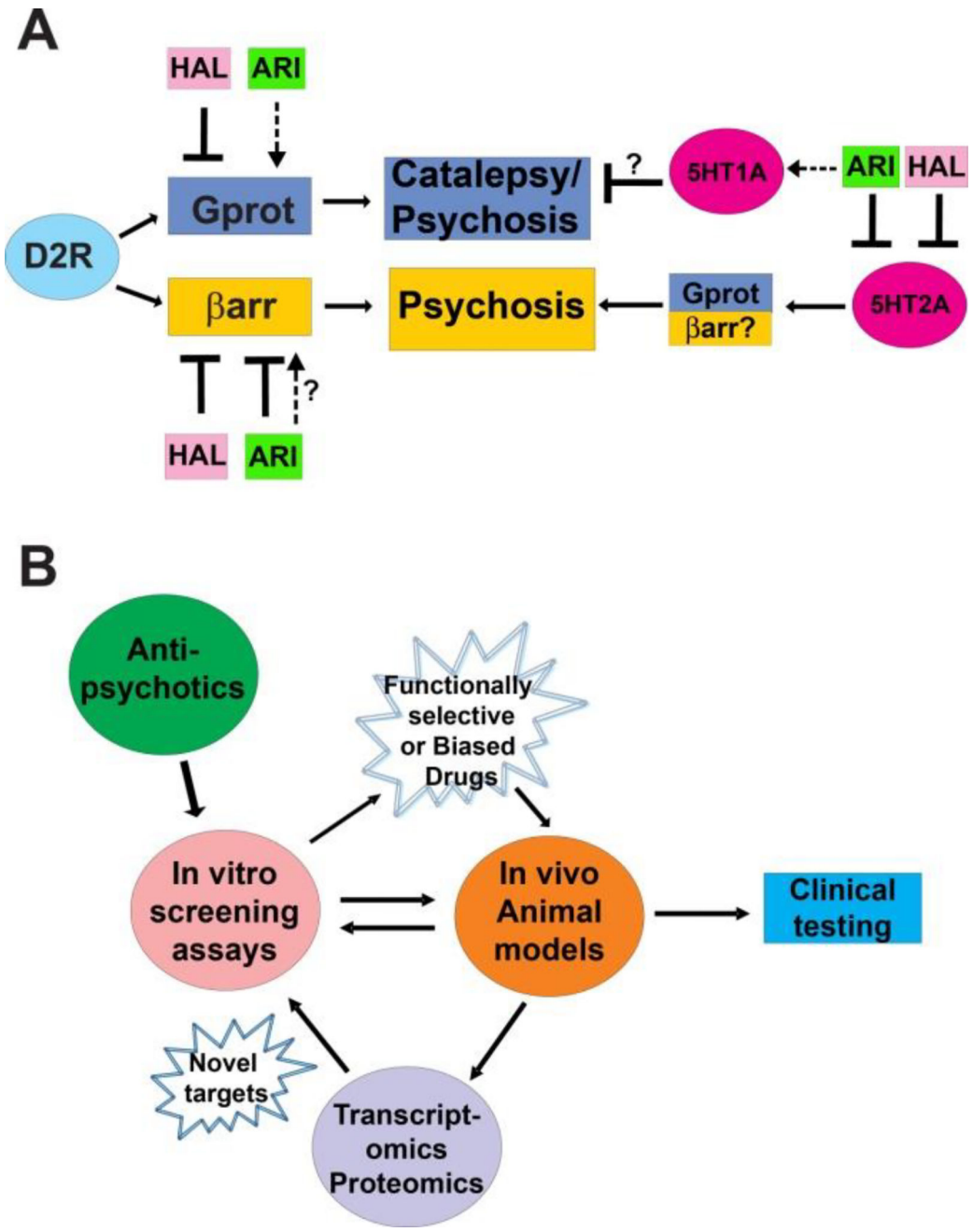

Figure 1.

A) Schematic representation of antipsychotic (APS) action on G-protein (Gprot) and β-arrestin (βarr) signaling pathways and its effect on catalepsy and psychosis. ⟶ Activation; ⊤ inhibition/antagonist; ⇣ partial agonist. Haloperidol (HAL) and aripiprazole (ARI) are used as representative typical and atypical APS, respectively. HAL and ARI act on D2 dopamine (D2R) and 5-HT2A serotonin receptors. Unlike HAL, ARI is a partial agonist at the D2-Gprot pathway and at 5-HT1A receptors, which might explain its non-cataleptic properties. Moreover, antagonist activity at the D2-βarr pathway and at 5-HT2A receptors might be largely responsible for reducing psychosis.

B) Schematic representation of the various integrated approaches used to decipher antipsychotic action including novel techniques in development.

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Table 1

Adapted and modified from Masri et al (39). EC_{50} and E_{max} of antipsychotics for both intrinsic and antagonist activity are presented. These in vitro data are compared to behavioral data in the last column.

Compounds	**Intrinsic activity for $G_{i/o}$ Pathway**		**Intrinsic activity for β-arrestin 2 pathway**		**Antagonistic activity for $G_{i/o}$ pathway**		**Antagonistic activity for β-arrestin 2 pathway**		**Inhibition of PCP or Amph locomotion in βarr2 signaling deficient mice**
	pEC_{50}	**E_{max} (%)**	**pEC_{50}**	**E_{max} (%)**	**K_B (nM)**	**E_{max} (%)**	**K_B (nM)**	**E_{max} (%)**	
Quinpirole	**8.25 ± 0.12**	**66.02 ± 1.05**	**7.01 ± 0.04**	**100 ± 1.01**	**N.A.**	**N.A.**	**N.A.**	**N.A.**	**N.A.**
Haloperidol	7.32 ± 0.39	−42.6 ± 4.5	N.A.	N.A.	0.28 ± 0.16	104 ± 4.5	0.12 ± 0.03	83.7 ± 2.5	**Y**[66], *a*
Clozapine	6.97 ± 0.52	−56.3 ± 9.8	N.A.	N.A.	>10,000	N.A.	67.7 ± 14.0	85.7 ± 8.2	**Y**[52],*a*
Aripiprazole	9.45 ± 0.24	49.0 ± 3.9	N.A.	N.A.	0.12 ± 0.05	29.9 ± 5.6	1.07 ± 0.32	69.6 ± 2.8	
Risperidone	9.25 ± 0.35	−16.4 ± 2.8	N.A.	N.A.	1.08 ± 0.9	96.3 ± 7.0	0.02 ± 0.01	83.0 ± 3.0	**Y** *a*

The behavioral data represent inhibition of either amphetamine (amph) or phencyclidine (PCP) induced locomotion in βarr2 signaling deficient mice (i.e βarr2−/− or D2GSK3β−/− mice);

*a*Urs et al Society for Neuroscience 2011 poster;

N.A not applicable; N=No inhibition, Y=lnhibition.