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Arrestin-3 Agonism at Dopamine D_3 Receptors Defines a Subclass of Second-Generation Antipsychotics That Promotes Drug Tolerance

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Abstract

BACKGROUND: Second-generation antipsychotics (SGAs) are frontline treatments for serious mental illness. Often, individual patients benefit only from some SGAs and not others. The mechanisms underlying this unpredictability in treatment efficacy remain unclear. All SGAs bind the dopamine D_3 receptor (D3R) and are traditionally considered antagonists for dopamine receptor signaling.

METHODS: Here, we used a combination of two-photon calcium imaging, in vitro signaling assays, and mouse behavior to assess signaling by SGAs at D3R.

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RESULTS: We report that some clinically important SGAs function as arrestin-3 agonists at D3R, resulting in modulation of calcium channels localized to the site of action potential initiation in prefrontal cortex pyramidal neurons. We further show that chronic treatment with an arrestin-3 agonist SGA, but not an antagonist SGA, abolishes D3R function through postendocytic receptor degradation by GASP1 (G protein–coupled receptor–associated sorting protein-1).

CONCLUSIONS: These results implicate D3R–arrestin-3 signaling as a source of SGA variability, highlighting the importance of including arrestin-3 signaling in characterizations of drug action. Furthermore, they suggest that postendocytic receptor trafficking that occurs during chronic SGA treatment may contribute to treatment efficacy.

Second-generation antipsychotics (SGAs) are important tools in the management of serious mental illness (SMI), including bipolar disorder, major depressive disorder, schizophrenia, and schizoaffective disorder (1,2). Each of these medications has unique dosing, pharmacokinetics, effect/side-effect profiles, and cost, and clinicians must often prescribe several drugs before finding a treatment regimen that suits individual patients (3,4). This approach primarily reflects an incomplete understanding of the molecular mechanisms underlying variable treatment efficacy in patients, even though these drugs have been in use for decades (5). All SGAs are antagonists or partial agonists for G-protein signaling at the dopamine D_2 receptor (D2R), and their clinical efficacy is thought to rely on their ability to block dopamine signaling at D2R (6,7). However, many SGAs also have high affinity for the dopamine D_3 receptor (D3R) (8), and positron emission tomography experiments in human patients that form the foundation of the D2R antagonism hypothesis of SGA efficacy did not distinguish between D2R and D3R occupancy (9). Though D3Rs are more sparsely expressed than D2Rs, they are highly enriched in brain regions known to be altered in SMI, including the striatum (10), islands of Calleja (11), hippocampus (12), and prefrontal cortex (PFC) (13), where they define a unique population of layer V pyramidal cells (14). Given these expression patterns in limbic systems, it is important to determine the effects of SGAs on D3R function (15).

Dopamine receptors, like most G protein–coupled receptors (GPCRs), not only engage G-proteins upon activation but also recruit other effectors, including arrestin-3 (β-arrestin-2) (16,17). Arrestin-3 recruitment both arrests the canonical G-protein signal and also scaffolds noncanonical kinase activity, including ERK (extracellular signal-regulated kinase)–1/2 (18). We recently demonstrated that ERK signaling via arrestin-3 is required for D3R to modulate calcium channel $Ca_V3.2$ at the axon initial segment (AIS) after agonist activation (19). There has been no previous assessment of SGA ability to recruit arrestin-3 to D3R, despite the importance of D3R in SGA activity. Here, we report that some clinically important SGAs are arrestin-biased agonists at D3R, promoting recruitment of arrestin-3 and ERK activation in the absence of G-protein activation. We further demonstrate that only the arrestin-biased SGAs modulate $Ca_V3.2$ activity in layer 5 neurons of the PFC. These findings provide a novel means by which SGAs can be divided into two functional classes based on their acute effects.

Importantly, the full therapeutic effect of SGAs often takes weeks of treatment. This suggests that mechanisms other than acute signaling contribute to drug effects. In addition

to its acute signaling roles, arrestin-3 also facilitates endocytosis (internalization) of GPCRs through interaction with protein components of the clathrin-coated pit (20,21). Following endocytosis, both D2R and D3R are targeted for degradation in the lysosome via interaction with GASP1 (GPCR-associated sorting protein-1) (22,23). Therefore, agonists that drive endocytosis decrease receptor surface levels over time, while conversely, antagonists that block both G-protein and arrestin-3 recruitment prevent dopamine-mediated endocytosis and thereby maintain receptor levels over time (24,25). We hypothesized that chronic treatment with an arrestin-biased SGA would decrease the number of functional D3Rs and would do so with a time-course more aligned with observed SGA treatment efficacy. We demonstrate here that chronic treatment with an arrestin-biased SGA eliminates the acute effects of D3R on AIS $Ca_V3.2$ channel function in wild-type (WT) mice but not in mice with conditional deletion of GASP1. We further demonstrate that mice treated chronically with an arrestin-3–biased SGA develop tolerance to the locomotor-inhibitory effects of the drug and that this tolerance is abolished in mice lacking GASP1 in D3R-expressing (D3+) neurons. These findings provide a new mechanistic framework for understanding the therapeutic actions of SGA drugs.

METHODS AND MATERIALS

Briefly, acute and chronic signaling engaged at D3Rs was assessed with molecular approaches in heterologous expression systems, then validated ex vivo using whole-cell electrophysiology and two-photon imaging and in vivo with behavior. Please see Supplemental Methods for details.

RESULTS

Quinpirole Modulates AIS Calcium Through D3R and Arrestin-3 in the PFC

The AIS is enriched with a number of ion channel classes, including $Ca_V3.2$ calcium channels that are the target of neuromodulation via D3Rs (14,26-30). D3R-dependent modulation hyperpolarizes steady-state voltage-dependent inactivation of $Ca_V3.2$ in a PKC (protein kinase C)- and ERK-dependent manner, leading to a reduction in high-frequency bursts of action potentials (APs) that depends in part on $Ca_V3.2$ activity (14,19,31). In cartwheel cells of the dorsal cochlear nucleus, we showed that this effect requires both D3R and noncanonical arrestin-3 signaling (Figure 1A) (19,31). We also reported previously that quinpirole, a D2/3R agonist, modulates $Ca_V3.2$ at the AIS, specifically in D3+ pyramidal cells in PFC (14). To examine whether this D3R-$Ca_V3.2$ modulation at the AIS in the PFC was also mediated by arrestin-3, we performed whole-cell current-clamp recordings from D3+ PFC pyramidal neurons in WT, D3R knockout (D3 KO), and arrestin-3 knockout (arrestin-3 KO) mice. D3+ neurons were identified either by fluorescent labeling of D3+ neurons in D3-Cre::Ai14 mice or by targeting neurons with specific intrinsic electrophysiological properties that allow for unambiguous identification of D3+ pyramidal cells (14). Using this approach (Figure 1B), we replicated our previous findings that AIS calcium was reduced by 30.4% ± 2.9% after 20 minutes of quinpirole application (Figure 1C, D) versus time-locked vehicle controls (Figure 1D). There was no quinpirole effect on AIS calcium in D3 KO mice or arrestin-3 KO mice (Figure 1D). Furthermore, inhibition

of GPCR kinase 2/3 with CMPD101 (30 μM) blocked quinpirole-induced AIS calcium modulation (Figure S1). Taken together, these data indicate that in the PFC, quinpirole-mediated modulation of AIS calcium in D3+ neurons requires arrestin-3 (Figure 1A).

Arrestin-3 Recruitment to D3R Is Both Ligand and PKC Dependent

Previous work has demonstrated that maximal endocytosis of D3R requires both receptor activation by agonist and phosphorylation of receptor by PKC (22,32). We hypothesized that arrestin-3 recruitment would also depend on both PKC activation and agonist binding. Consistent with this hypothesis, D3R-dependent modulation of AIS $Ca_V3.2$ is both ligand and PKC dependent (19,31). We therefore examined whether PKC activity, concomitant with D3R agonist, is required for arrestin-3 recruitment to D3R. To test this, we visualized changes in the distribution of cytosolic GFP (green fluorescent protein)–tagged arrestin-3 relative to FLAG-tagged D3R on the plasma membrane in human embryonic kidney (HEK)-293 cells under a series of treatment conditions (Figure 2). In untreated cells and cells treated with only quinpirole or only the PKC activator PMA (phorbol-12-myristate-13-acetate), arrestin-3 remained diffusely distributed and did not colocalize with D3R at the plasma membrane (Figure 2B, C). By contrast, arrestin-3 was redistributed close to surface D3R in cells treated with quinpirole plus PMA (Figure 2B, C). Note that this time point preceded any significant receptor endocytosis. These results demonstrate that, in HEK-293 cells, quinpirole is an agonist for arrestin-3 recruitment only with coincident PKC activation.

Some SGAs Recruit Arrestin-3 to D3R

There is mounting evidence that different GPCR drugs can stabilize distinct receptor conformations that preferentially engage either G-protein or arrestin-3 (33). Based on this, we hypothesized that some SGAs could promote arrestin-3 recruitment to D3R even in the absence of G-protein activation. To examine this hypothesis, we performed the arrestin-3–GFP redistribution experiments (Figure 2), but with 4 commonly prescribed SGAs: roxindole, aripiprazole, quetiapine, and clozapine. Consistent with previous reports, among these ligands, only roxindole produced any G-protein stimulation from D3R. Despite this, we found that three of these SGA ligands—including roxindole, but also quetiapine and aripiprazole—recruited arrestin-3 (Figure 3A, left; 3B, C). By contrast, clozapine did not produce a change in arrestin-3 localization (Figure 3A, right; 3B, C). These results demonstrate that some SGAs promote arrestin-3 recruitment even in the absence of G-protein signaling (quetiapine, aripiprazole), while others engage neither G-protein nor arrestin-3 (clozapine) at D3R.

Some SGAs Promote ERK Phosphorylation Through D3R and Arrestin-3

We previously showed that both arrestin-3 and ERK are required to modulate $Ca_V3.2$ (19). ERK phosphorylation by some GPCRs has been shown to occur not only through G-protein activation but also through arrestin/ERK scaffolding independent of G-protein (34). Therefore, we examined the ability of SGAs that did and did not recruit arrestin-3 to promote ERK phosphorylation in HEK-293 cells expressing D3R (Figure 4A). Given the PKC dependence shown in Figures 2 and 3, all experiments were performed in either the absence or presence of PKC activation by carbachol, an agonist for the G_q-coupled muscarinic receptors that are expressed endogenously in HEK-293 cells. This approach

allowed us to titrate a dose of carbachol that stimulated little ERK phosphorylation on its own but synergized with quinpirole (Figure 4C). ERK phosphorylation by carbachol + quinpirole was transient and returned to baseline within 30 minutes (Figure S2). We then assessed whether SGAs could induce ERK phosphorylation in the presence of carbachol. Importantly, we found that the same SGAs that recruit arrestin-3 (Figure 3) also promote ERK phosphorylation but only when PKC is activated (Figure 4C). These data suggest that some SGAs (e.g., aripiprazole, quetiapine) can promote ERK phosphorylation even in the absence of any D3R-mediated G-protein signaling (Figure 4B), classifying them as arrestin-biased agonists. Others (e.g., roxindole) are both G-protein (Figure 4B) and arrestin-3 agonists, and others still (e.g., clozapine) are antagonists for both.

Arrestin-Biased SGAs Modulate AIS Calcium in D3+ Pyramidal Cells in the PFC

We established that arrestin-3 is necessary for quinpirole-activated D3R to modulate AIS calcium in PFC (Figure 1). However, quinpirole is both a G-protein and an arrestin-3 agonist at D3R, which precludes the ability to determine whether arrestin-3 recruitment alone is sufficient to modulate AIS calcium. The SGAs that promote arrestin-3 recruitment (Figure 3) and ERK phosphorylation (Figure 4C), even in the absence of G-protein activation (Figure 4B), are ideal tools to address this question. Recordings were made from D3+ PFC neurons (Figure 1B), and AIS calcium transients were imaged in the presence of vehicle, quinpirole (positive control), or SGA (Figure 5A). These transients were stable for interleaved control cells treated with vehicle for 20 minutes, while 20 minutes of roxindole, aripiprazole, or quetiapine produced a ~30% reduction in AIS calcium compared with baseline, a magnitude comparable to that observed with quinpirole (Figure 5B). By contrast, clozapine did not modulate AIS calcium transients (Figure 5B).

SGAs have a high affinity for D3R but also bind to other GPCRs. To determine whether the AIS calcium modulation by SGAs occurs via D3R and to demonstrate that SGA modulation of $Ca_V3.2$ is arrestin-3 dependent, we assessed AIS calcium modulation by quetiapine in slices from both D3 KO and arrestin-3 KO mice. There was no change in AIS calcium with quetiapine treatment in either genotype (Figure 5C).

To test whether these effects of some SGAs are unique to the PFC, we also performed whole-cell recordings from D3+ dorsal hippocampal CA1 pyramidal cells (Figure 5D). As in the PFC, both quinpirole and quetiapine reduced AIS calcium (Figure 5E). Taken together, these results suggest that some SGAs are arrestin-biased agonists at D3R and can modulate calcium at the AIS in the PFC and hippocampus, even in the absence of G-protein activation.

Mice Treated Chronically With Quetiapine, but Not Clozapine, Develop Tolerance to the Locomotor-Inhibitory Effects of the Drug

SGAs can cause locomotor effects, including dystonia and parkinsonism in humans (35,36) and locomotor inhibition in mice (37). While both effects are traditionally thought to be mediated through D2R (36,38), mounting evidence suggests that D3R may also be important for the locomotor effects of SGAs (39-50). Individual SGAs have variable degrees of locomotor side effects. We next tested the hypothesis that ability to act as an arrestin-3 agonist contributes to this variability by assessing the locomotor-inhibitory effects of an

SGA that did (quetiapine) and did not (clozapine) recruit arrestin-3. Both quetiapine (15 mg/kg) and clozapine (4 mg/kg) inhibited locomotor activity (Figure 6A), indicating that arrestin-3 recruitment to D3R is not necessary for locomotor inhibition by these SGAs.

While both quetiapine and clozapine inhibited locomotion acutely, the optimal treatment efficacy of SGAs and equilibrium of effect/side-effect profile requires weeks of treatment. Hence, we also examined the effects of quetiapine and clozapine on locomotion following 21 days of drug treatment (15 mg/kg quetiapine or 4 mg/kg clozapine 1× per day). Remarkably, on day 21, quetiapine no longer reduced locomotion, while clozapine's effect was indistinguishable from that observed on day 1 (Figure 6B). These results demonstrate that mice treated chronically with quetiapine develop tolerance to the locomotor-inhibitory effects of the drug, while animals treated with clozapine do not.

Chronic Treatment With Quetiapine, but Not Clozapine, Eliminates D3R-Ca_V3.2 Signaling at the AIS

We next examined whether we could observe drug tolerance on a single-cell level. Mice were treated for 21 days with quetiapine or clozapine (as in Figure 6). At the end of this regimen, AIS calcium transients were assessed in D3+ pyramidal cells in the PFC. In mice treated with quetiapine for 21 days, quinpirole had no effect (Figure 6C, D). By contrast, 21-day treatment with clozapine (or vehicle) did not interfere with quinpirole-mediated modulation of AIS Ca_V3.2 (Figure 6C, D). These results suggest that chronic treatment with an arrestin-biased SGA, but not an SGA with no arrestin-3 engagement, causes a loss of D3R function at the AIS in the PFC. Furthermore, these results, together with the locomotor tolerance phenotype (Figure 6A, B), divide SGA function at D3R into 2 classes.

Both Quetiapine-Induced Loss of D3R-Ca_V3.2 Modulation and Development of Locomotor Tolerance Are Mediated by Postendocytic Sorting of D3R by GASP1

Recruitment of arrestin-3 to D3R not only arrests the G-protein signal and scaffolds ERK signaling but also promotes receptor endocytosis and sorting of D3R to the lysosome for degradation through interaction with GASP1 (22) (Figure 7A). To examine whether this sorting mechanism contributed to tolerance, we generated mice with selective disruption of the GASP1 gene only in cells expressing D3R by crossing D3-Cre driver mice to conditional floxed GASP1 mice (D3 GASP1 cKO). We then treated these D3 GASP1 cKO mice for 21 days with quetiapine (15 mg/kg) (as in Figure 6) and measured AIS calcium modulation by quinpirole in D3+ PFC cells. D3 GASP1 cKO mice treated with quetiapine for 21 days showed intact quinpirole-mediated modulation of AIS calcium (Figure 7B, C) in stark contrast to WT mice (Figure 6C, D). To test whether GASP1-mediated postendocytic sorting of D3R was also necessary for tolerance to the locomotor-inhibitory effects of quetiapine, we treated D3 GASP1 cKO mice with quetiapine for 21 days (Figure 7D, E). Unlike WT mice or floxed GASP1 mice not crossed to D3-Cre (21-day vehicle: median 33.9 m, interquartile range 20.3–45.6 m, $n = 4$ mice; 21 days quetiapine: median 24.8 m, interquartile range 16.1–39.6 m, $n = 4$ mice, $p = .486$, Mann-Whitney test), D3 GASP-cKO mice did not develop tolerance to the locomotor effects of quetiapine (Figure 7D, E).

DISCUSSION

Here, we demonstrate that some SGAs are agonists for arrestin-3 signaling at D3R in the absence of G-protein activity. In D3+ neurons of the PFC, acute slice treatment with these arrestin-biased SGAs results in reduced AP-associated AIS calcium influx, demonstrating an acute mechanism of action of a subset of SGAs in addition to their role as D2R and D3R G-protein antagonists. We also establish that chronic treatment with an SGA that recruits arrestin-3 to D3R results in GASP1-mediated postendocytic sorting, presumably to the lysosome as previously shown (22), and results in subsequent reduction of both D3R function at the AIS in D3+ PFC pyramidal cells and SGA-induced locomotor inhibition. The receptor trafficking–mediated loss of D3R at sites of endogenous neuromodulation as a consequence of chronic SGA exposure may be an important aspect of treatment and could help explain why prolonged SGA treatment is necessary for full clinical efficacy.

Arrestin-3 Signaling at D3R

Several studies have focused on arrestin-3 signaling at D2R (51-53), and, in the context of antipsychotic drugs, aimed to leverage G-protein or arrestin-3 signaling to maximize therapeutic benefits while minimizing side effects (17,54-59). Here, we show that select SGAs can also engage in arrestin-3 signaling at D3R. We found that in HEK-293 cells, some SGAs, with coincident PKC activation, recruit arrestin-3 to D3R. Notably, aripiprazole recruits arrestin-3 to D3R (Figure 3), but not to D2R (56) except when GRK2 is overexpressed (54), and promotes G-protein signaling at D2R but not D3R. Aripiprazole is thus a G protein–biased agonist at D2R and an arrestin-biased agonist at D3R. This provides evidence that, even though D2R and D3R are highly homologous, SGAs do not engage the two receptors identically.

Importantly, arrestin-3 recruitment to D3R is sufficient to scaffold ERK phosphorylation even in the absence of G-protein signaling (Figure 4). Specifically, aripiprazole and quetiapine promote increased phospho-ERK levels (Figure 4C), even though they do not promote G-protein activation from D3R (Figure 4B). We found previously that quinpirole modulates AIS-localized $Ca_V3.2$, hyperpolarizing the voltage dependence of steady-state inactivation (19). This modulation is both arrestin-3 and ERK dependent (14,19,31). We tested here whether arrestin-biased SGAs at D3Rs could activate this signaling pathway. We found that only the SGAs that recruit arrestin-3 in HEK-293 cells reduce AIS calcium transients at timescales consistent with the timing of arrestin-3–dependent ERK phosphorylation (e.g., minutes rather than seconds following ligand application).

Kim *et al.* (51) found previously that arrestin-3 did not strongly translocate to D3R in heterologous cells, presumably because there was no concomitant activation of PKC. In HEK-293 cells, we achieved PKC activation and ERK phosphorylation by either stimulating PKC directly or activating a G_q-coupled GPCR. In neurons, PKC may be activated due to ongoing AP activity, as this can induce release of calcium from intracellular stores (30). In this way, AIS D3R signaling may serve as a coincidence detector for ligand binding and ongoing activity. If this were the case, receptors bound by an arrestin-recruiting dopaminergic ligand would promote signaling only if the neuron was recently firing APs at levels sufficient to activate PKC. Under endogenous conditions, this arrestin-recruiting

ligand would be dopamine, but dopamine could be substituted with an arrestin-biased SGA during treatment.

Studies of some other GPCRs have indicated that arrestin-3 signaling cannot occur in the absence of G-protein activity (60). For D3R-arrestin-3-$Ca_V3.2$ modulation at the AIS, however, our results indicate that D3R can signal through arrestin-3 in a G protein–independent manner because SGAs that do not engage G-protein nevertheless produce channel modulation. Even in HEK-293 cells, D3R ligands that do not promote G-protein activity recruit arrestin-3 and promote ERK phosphorylation, indicating that this signaling mechanism could be conserved across many cell types. While other groups have shown GPCR modulation of ion channels at the AIS (26,61,62), D3R's inhibition of $Ca_V3.2$ is, to our knowledge, the first example of a GPCR directly inhibiting a channel solely through an arrestin-3 effector. This finding could have significant implications if it also occurs with other key GPCR drug targets. Future studies should examine whether drugs considered to be antagonists could likewise signal via arrestin-3 to channels or other downstream effectors.

Here, we show that acute application of an arrestin-biased SGA to an ex vivo slice of mouse PFC modulates $Ca_V3.2$ at the AIS via D3R and that chronic treatment of mice with the same SGA results in loss of D3R and this modulatory effect. $Ca_V3.2$ channels play a critical role in the generation of high-frequency bursts of APs in many cell classes (63). This modulatory pathway decreases the number of $Ca_V3.2$ channels that can be recruited during APs, suppressing burst generation (14,31,64). Thus, arrestin-3–biased SGAs may acutely act to change bursting properties in D3+ neurons. However, following chronic treatment and loss of membrane D3R, neurons may no longer alter their firing patterns based on the presence or absence of dopaminergic input. In the PFC, this could be one potential mechanism of SGA action and one that differentiates SGAs into two distinct classes: those that do and do not recruit arrestin-3 and promote loss of D3R function at this site.

Because D3+ pyramidal cells are largely a distinct population of cells from those expressing D1R or D2R in the PFC, we could isolate SGA-mediated arrestin-3 recruitment at D3R specifically (14,65,66). The role of D3R signaling in other relevant brain regions, including the nucleus accumbens, islands of Calleja, and lateral septum, remains unclear (67-71). In future studies, it will be critical to understand fully how D3R regulates neuronal function via arrestin-dependent or more canonical signaling pathways, as well as to understand more clearly how these signaling pathways are affected by SGAs (70,72-74).

D3R as a Target for SGAs

While modulation of $Ca_V3.2$ is an acute SGA effect, antipsychotic drugs often take weeks to months to reach maximal therapeutic benefit. Here, we report two distinct effects of chronic treatment with an arrestin-biased SGA that are mediated by postendocytic degradation of D3R by GASP1: 1) the ability of dopaminergic drugs, and by extension dopamine, to modulate calcium influx at the AIS is lost and 2) mice become tolerant to the locomotor-inhibitory effects of drug. Specifically, we show that chronic treatment with the arrestin-3 agonist SGA quetiapine causes a loss of D3R function at the AIS (Figure 6). This loss is prevented in mice with a disruption of the sorting protein GASP1 specifically in D3+ neurons (Figure 7). Additionally, we show that chronic treatment with quetiapine results in

loss of drug-mediated locomotor suppression (Figure 6), an effect that was also eliminated in D3 GASP1 cKO mice (Figure 7). This result highlights the importance of D3R in the locomotor side effects of SGAs and, by extension, the effect/side-effect profile of SGAs. Together these data directly implicate postendocytic sorting as a key mechanism controlling the amount of functional D3R in multiple circuits, including cognitive and motor circuitry. Importantly, this trafficking mechanism regulates receptor function in a ligand-dependent manner, independent of messenger RNA expression, potentially reconciling why people living with SMI show altered receptor protein levels but small, if any, changes in dopamine receptor gene expression (75).

Implications for Patients

In 2017, roughly 11 million adults, or 4.2% of the population, in the United States had an SMI diagnosis (76). Recently, several meta-analyses sought to elucidate which antipsychotic drugs benefit patients the most (77,78). Patients living with SMI often go through a number of drug protocols before finding an effective strategy through a protracted and stochastic process. A complete understanding of the molecular mechanisms underlying the variable acute and chronic actions of these drugs could help inform a more streamlined approach for an individual patient.

Here, we show that SGAs can be separated into two classes based on their D3R-mediated arrestin-3 signaling. SGAs that recruit arrestin-3 affect D3+ neurons by modulating calcium at the AIS acutely and by reducing D3R levels at the AIS over time. Historically, GPCR-targeting drugs have been characterized solely for their ability to alter G-protein activity. More recently, the role of arrestin-3–mediated signaling in drug response has come to light. Importantly, arrestin-3 not only arrests G-protein signaling and scaffolds signal transduction to other effectors but also promotes receptor endocytosis. Therefore, when considering the implications of arrestin-3 engagement, it is important to account for not only acute signaling but also how engagement on a longer timescale can change receptor distribution and surface expression through endocytosis and postendocytic degradation. This may be particularly relevant for GPCR drugs, such as SGAs, that have rapid pharmacokinetics, reaching the brain within minutes, but that take days or weeks to reach maximal efficacy.

SMI diagnoses describe constellations of symptoms that vary from patient to patient, and the genetic and environmental factors underlying SMI are complex (79). Therefore, two individuals with a similar SMI diagnosis may have different underlying etiologies. Moving forward, it would be helpful to track patient diagnosis and symptoms and map drug effect/side-effect profiles onto whether an arrestin-3–biased SGA was or was not therapeutically beneficial. We posit that, for some patients, treatment with an arrestin-biased ligand to reduce D3R levels would be the most efficacious. For others, treatment with an SGA that blocks dopamine-mediated arrestin-3 recruitment, receptor endocytosis, and postendocytic degradation to increase membrane D3R levels may be more effective. In conclusion, the findings here contribute to a mechanistic understanding of how D3R signaling can vary across different effectors and brain regions and could inform a more personalized approach to treatment with SGAs. D3R has also been suggested as a potential therapeutic target for

Parkinson's disease and substance use disorder (80,81). Hence, our findings may inform the development of D3R-selective ligands, either biased or not, for multiple indications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SS, EL, AH, KJB, and JLW were responsible for conceptualization; SS, EL, CMK, AH, KJB, and JLW were responsible for methodology; SS, EL, CMK, KJB, and JLW were responsible for formal analysis; SS, EL, CMK, AH, KJB, and JLW were responsible for investigation; SS was responsible for writing the original draft; SS, EL, AH, KJB, and JLW were responsible for reviewing and editing; and KJB and JLW were responsible for supervision, project administration, and funding acquisition.

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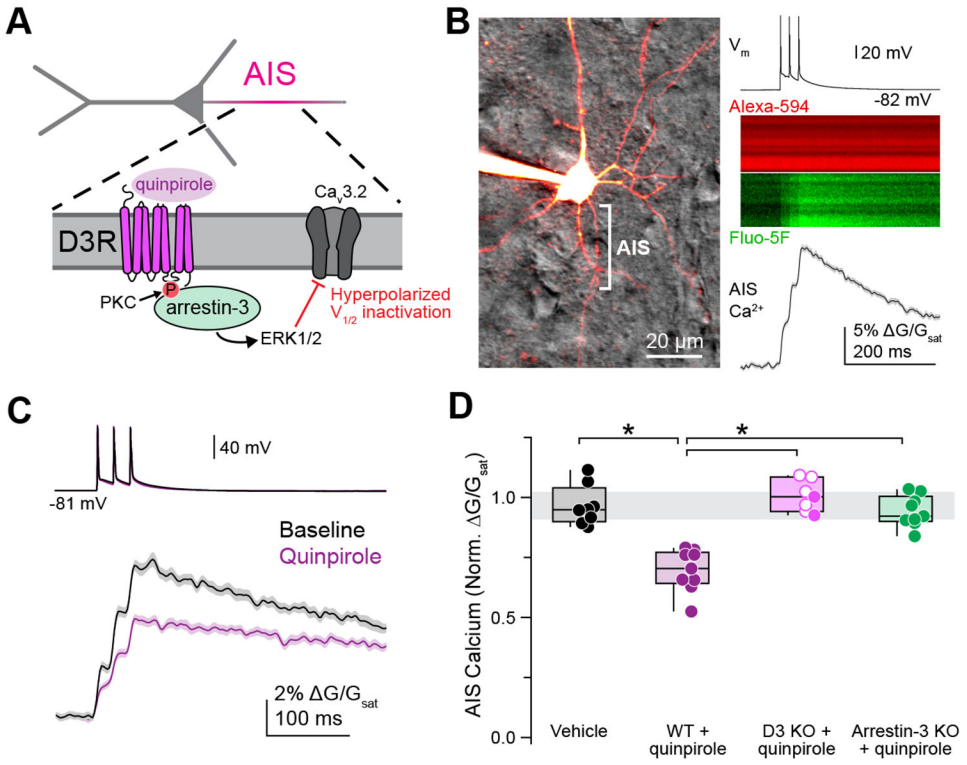

Figure 1.

Quinpirole modulates AIS calcium through D3R and arrestin-3 in the prefrontal cortex. **(A)** Schematic of quinpirole modulation of calcium at the AIS of a D3+ pyramidal cell in prefrontal cortex. **(B)** Left, two-photon laser-scanning microscopy z-stack of a D3+ pyramidal neuron visualized with Alexa Fluor 594. AIS is denoted with bracket. Right, example linescan of AIS calcium averaged over 40 trials. Action potentials were evoked with somatic current injection (3 at 50 Hz, 2 nA, 2 ms per stimulus). Linescan data displayed as mean ± SEM. **(C)** Representative effect of quinpirole on AIS calcium in a D3+ neuron, averaged over 20 trials per condition. Baseline, black; quinpirole, purple. **(D)** Peak AIS calcium transient amplitude normalized to baseline for vehicle and quinpirole in WT, D3 KO, and arrestin-3 KO mice. Vehicle controls include all 3 genotypes. For D3 KO, open circles denote previously published data (14). Gray bar represents 95% confidence interval of control data. Vehicle: median normalized peak $\Delta G/G_{sat}$ = 94.8% of baseline, IQR 89.9%–104.0%, n = 8 cells from 6 mice; WT + quinpirole: 70.4% of baseline, IQR 64.2%–77.2%, n = 9 cells from 7 mice, p < .001; D3 KO + quinpirole: 100.3% of baseline, IQR 94.2%–108.5%, n = 7 cells from 4 mice, p = .001; arrestin-3 KO + quinpirole: 92.2% of baseline, IQR 90.0%–100.5%, n = 9 cells from 5 mice, p < .001. Kruskal-Wallis test with Mann-Whitney U test post hoc (Holm-Šídák correction). AIS, axon initial segment; D3+, D3R-expressing; D3R, D_3 receptor; ERK1/2, extracellular signal-regulated kinase-1/2; IQR, interquartile range; KO, knockout; Norm., normalized; PKC, protein kinase C; WT, wild-type.

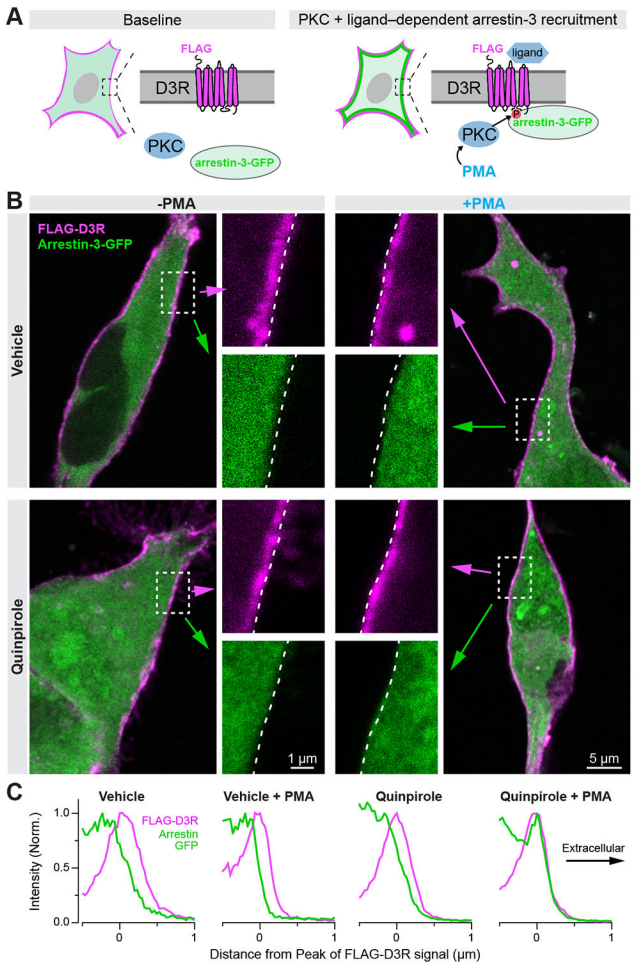

Figure 2.

Arrestin-3 recruitment to D3R is both ligand and PKC dependent. **(A)** Schematic showing that D3R ligand and PKC activation are both necessary to recruit arrestin-3 to D3R in HEK-293 cells. **(B)** Sample images of HEK-293 cells expressing FLAG-D3R and arrestin-3–GFP. Cells were treated with quinpirole (bottom row) and/or PMA (right column). Dotted lines in insets denote edge of cell membrane. **(C)** Quantification of images in **(B)** one region per condition, showing locations of peak FLAG-D3R and arrestin-3–GFP signal. FLAG-D3R in pink, arrestin-3–GFP in green. D3R, D_3 receptor; GFP, green fluorescent protein; HEK, human embryonic kidney; Norm., normalized; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate.

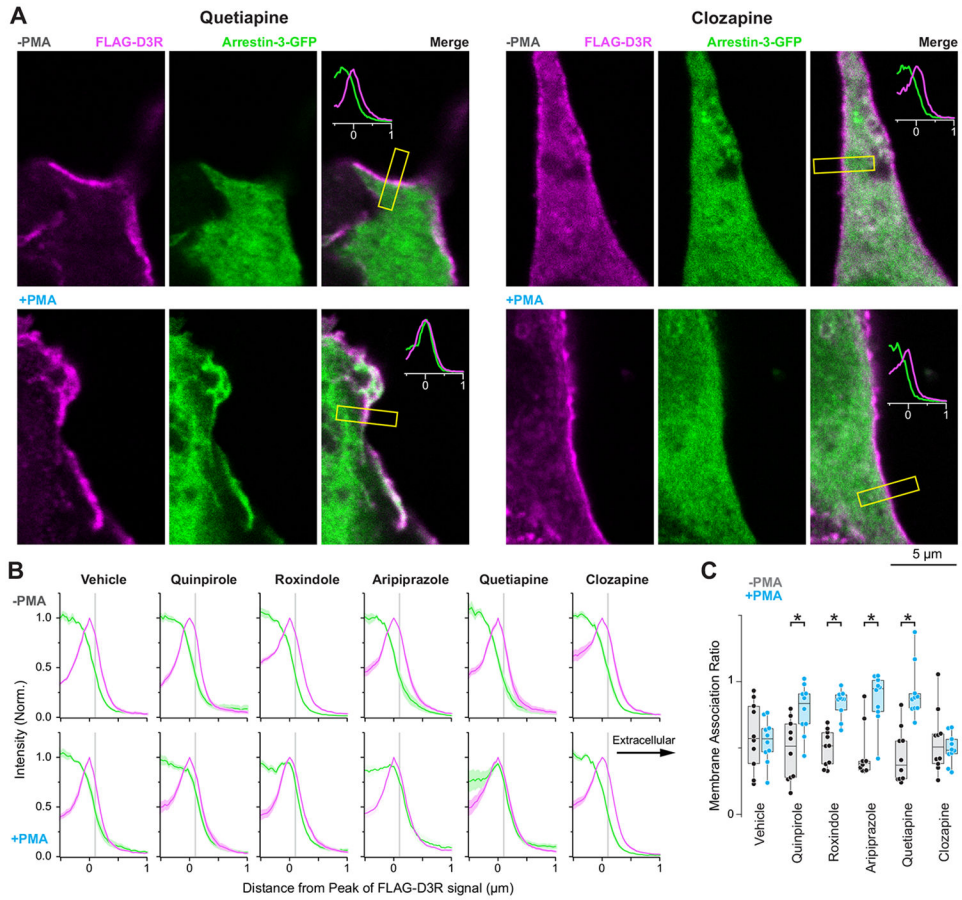

Figure 3.

Some second-generation antipsychotics recruit arrestin-3 to D3R. **(A)** Example HEK-293 cells expressing FLAG-D3R and arrestin-3–GFP. Cells were treated either with drug alone (quetiapine or clozapine) (top row) or with drug and PMA (bottom row). Yellow boxes denote regions of analysis. **(B)** Quantification of peak arrestin-3 signal relative to D3R signal for each second-generation antipsychotic without (top) or with (below) PMA to activate protein kinase C. Each line profile is mean ± SEM. FLAG-D3R in pink, arrestin-3–GFP in green. **(C)** Quantification of peak arrestin-3 signal relative to peak D3R signal (membrane association ratio), calculated at the gray bar in **(B)**. Black, drug alone; blue, drug plus PMA. Circles represent individual line profiles, 2 regions per cell, 5 cells per condition. *$p < .05$, Mann-Whitney test. No change with vehicle or clozapine. D3R, D_3 receptor; GFP, green fluorescent protein; Norm., normalized; PMA, phorbol-12-myristate-13-acetate.

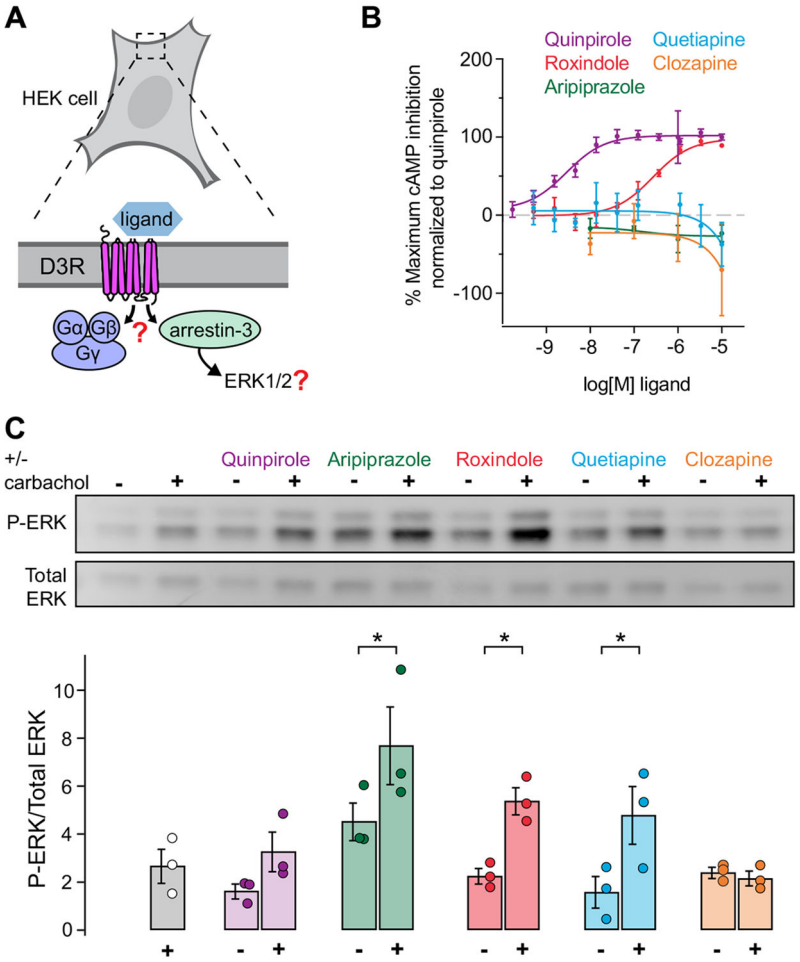

Figure 4.

Some second-generation antipsychotics promote ERK phosphorylation through D3R and arrestin-3. **(A)** Schematic of experimental question: do second-generation antipsychotics promote ERK phosphorylation? **(B)** Percent maximum cAMP inhibition, normalized to quinpirole E_{max}, as a readout of G-protein signaling for each drug. **(C)** (Top) Representative immunoblot of P-ERK and total ERK after 5-minute drug treatment, with or without carbachol to activate PKC, in HEK-293 cells stably expressing D3R. (Bottom) Quantification of P-ERK/total ERK across multiple experiments ($n = 3$). Data displayed as mean ± SEM. *$p < .05$ compared with drug alone, one-way analysis of variance with Holm-Šídák multiple comparisons test. cAMP, cyclic adenosine monophosphate; D3R, D_3 receptor; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; P-ERK, phosphorylated ERK.

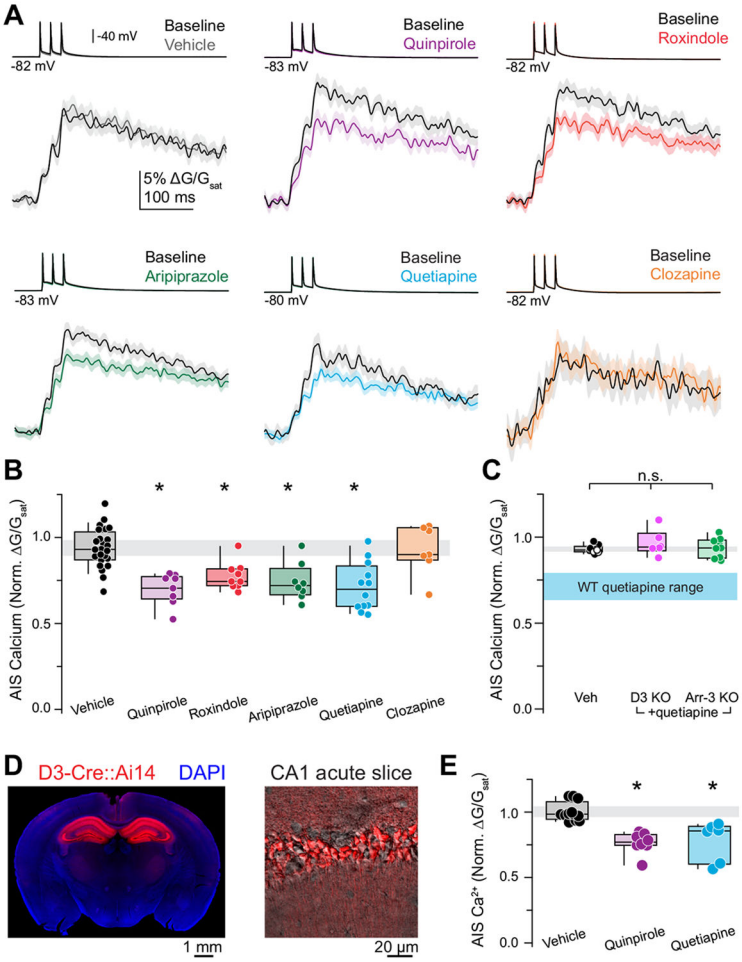

Figure 5.

Arrestin-biased second-generation antipsychotics modulate AIS calcium in D3+ pyramidal cells in the prefrontal cortex. **(A)** Representative effects of time-locked vehicle control or drug after 20 minutes, averaged across 20 trials per condition. Line scan data displayed as mean ± SEM. Baseline, black; drugs, other colors. **(B)** Data summarizing the effects of quinpirole and clinically relevant second-generation antipsychotics on AIS calcium. Gray bar represents 95% CI of control data. Vehicle: 93.1% of baseline, IQR 86.9%–103.3%, n = 24 cells from 21 mice; quinpirole: 70.4% of baseline, IQR 64.2%–77.2%, n = 9 cells from 7 mice, $p < .001$ (same data as in Figure 1); roxindole: 74.4% of baseline, IQR 71.9%–81.7%, n = 9 cells from 6 mice, $p < .001$; aripiprazole: 72.1% of baseline, IQR 66.7%–82.0%, n = 8 cells from 6 mice, $p = .002$; quetiapine: 69.9% of baseline, IQR 59.9%–83.5%, n = 12 cells from 6 mice, $p < .001$; clozapine: 90.0% of baseline, IQR 86.8%–105.6%, n = 7 cells from 6 mice, $p = .944$. Kruskal-Wallis test with Mann-Whitney U test post hoc (Holm-Šídák correction). Note: for clarity in the figure, we only indicated significant comparisons in relation to vehicle-treated cells. **(C)** Data summarizing the lack of effect of quetiapine on AIS calcium in D3 KO and arrestin-3 KO mice. Vehicle data include D3 KO (open circles) and arrestin-3 KO (closed circles) mice. Gray bar represents 95% CI of vehicle data from D3 KO and arrestin-3 KO animals, while cyan bar represents 95% CI of

quetiapine data in WT animals. Vehicle: 92.5% of baseline, IQR 91.4%–94.6%, $n = 9$ cells from 7 mice; D3 KO: 94.2% of baseline, IQR 92.1%–102.3%, $n = 6$ cells from 3 mice, $p = .213$; arrestin-3 KO: 93.7% of baseline, IQR 87.8%–98.3%, $n = 9$ cells from 4 mice. $p = .480$, Kruskal-Wallis test with Mann-Whitney U test post hoc (Holm-Šídák correction). **(D)** Left, coronal section of a D3-Cre::Ai14 mouse showing D3+ pyramidal and granule cells. Right, two-photon laser-scanning microscopy image showing D3+ pyramidal cells in dorsal CA1. **(E)** Data summarizing the effects of quinpirole and quetiapine on AIS calcium in D3+ dorsal hippocampal CA1 pyramidal cells. Gray bar represents 95% CI of data from vehicle-treated cells. Vehicle: 98.4% of baseline, IQR 94.5%–107.8%, $n = 12$ cells from 11 mice; quinpirole: 77.0% of baseline, IQR 74.6%–82.8%, $n = 8$ cells from 6 mice, $p < .001$; quetiapine: 85.6%, IQR 85.1%–96.4%, $n = 6$ cells from 5 mice, $p = .002$. Kruskal-Wallis test with Mann-Whitney U test post hoc (Holm-Šídák correction). AIS, axon initial segment; D3+, D_3 receptor–expressing; IQR, interquartile range; KO, knockout; Norm., normalized; n.s., not significant; Veh, vehicle; WT, wild-type.

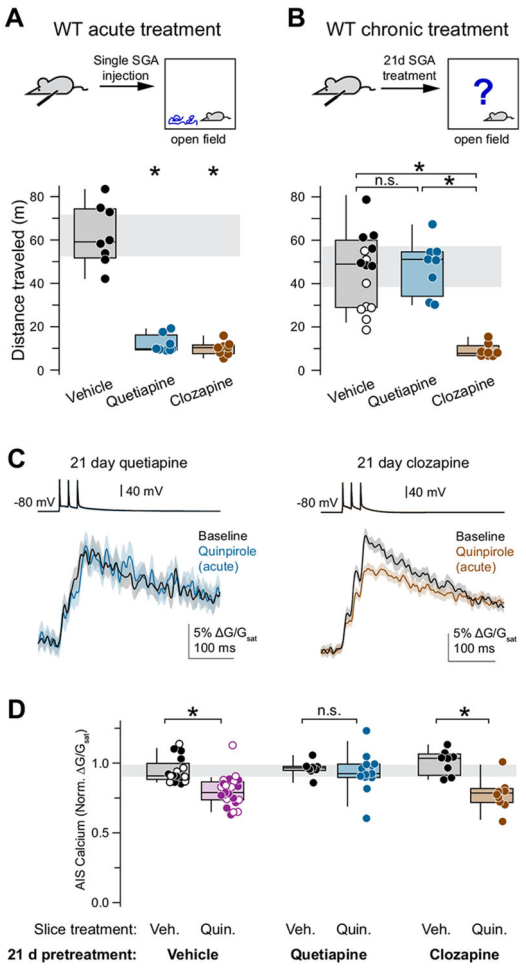

Figure 6.

Mice treated chronically with quetiapine, but not clozapine, develop tolerance to locomotor-inhibitory effects of the drug. **(A)** (Top) Mice were injected subcutaneously with drug or vehicle and immediately placed in the locomotor box, and distanced traveled was recorded for 60 minutes. (Bottom) Total distance traveled for each treatment condition. Gray bar represents 95% CI of vehicle-treated mice. Vehicle: 59.1 m, IQR 51.6–74.3 m, $n = 8$ mice; quetiapine: 9.81 m, IQR 9.32–16.20 m, $n = 8$ mice, $p < .001$; clozapine: 10.4 m, IQR 7.6–11.6 m, $n = 8$ mice, $p < .001$. **(B)** (Top) Same as **(A)** except that mice were pretreated with daily injections of drug or vehicle for 21 days. On day 21, they were placed in the locomotor box immediately following drug injection, and distance traveled was recorded for 60 minutes. (Bottom) Total distance traveled for each treatment condition. Open circles indicate clozapine vehicle control, while closed circles indicate quetiapine vehicle control. Gray bar represents 95% CI of vehicle-treated mice. Vehicle: 49.0 m, IQR 29.0–60.0 m, $n = 16$ mice; quetiapine: 51.1 m, IQR 34.1–54.6 m, $n = 8$ mice, compared with vehicle $p = .782$; clozapine: 7.9 m, IQR 6.8–11.1 m, $n = 8$ mice, compared with vehicle and quetiapine $p < .001$. Kruskal-Wallis test with Mann-Whitney U test post hoc (Holm-Šídák correction). **(C)** Representative examples showing effect of quinpirole on AIS calcium transients in mice treated with quetiapine, left, blue, or clozapine, right, brown. Data shown averaged

across 20 trials per condition. Line scan data displayed as mean ± SEM. Baseline, black; quinpirole, blue or brown. **(D)** Summary data comparing vehicle or quinpirole effect on AIS calcium in animals treated for 21 days with vehicle, quetiapine, or clozapine. Open circles indicate clozapine vehicle control, while closed circles indicate quetiapine vehicle control. Gray bar represents 95% CI of control data. Twenty-one-day vehicle treatment, vehicle slice treatment: 90.9% of baseline, IQR 88.3%–99.8%, $n = 17$ cells from 12 mice; 21-day vehicle treatment, quinpirole slice treatment: 78.8% of baseline, IQR 73.7%–86.5%, $n = 23$ cells from 13 mice, $p < .001$; 21-day quetiapine treatment, vehicle slice treatment: 96.5% of baseline, IQR 94.7%–97.7%, $n = 8$ cells from 6 mice; 21-day quetiapine treatment, quinpirole slice treatment: 92.5% of baseline, IQR 89.8%–99.3%, $n = 13$ cells from 6 mice, $p = .538$; 21-day clozapine treatment, vehicle slice treatment: 103.5% of baseline, IQR 91.3%–106.6%, $n = 8$ cells from 4 mice; 21-day clozapine treatment, quinpirole slice treatment: 78.5% of baseline, IQR 71.7%–81.7%, $n = 10$ cells from 6 mice, $p = .001$. Kruskal-Wallis test with Mann-Whitney U test post hoc (Holm-Šídák correction). Note: for clarity, we only indicate significant comparisons within drug. AIS, axon initial segment; IQR, interquartile range; Norm., normalized; n.s., not significant; Quin., quinpirole; SGA, second-generation antipsychotic; Veh., vehicle; WT, wild-type.

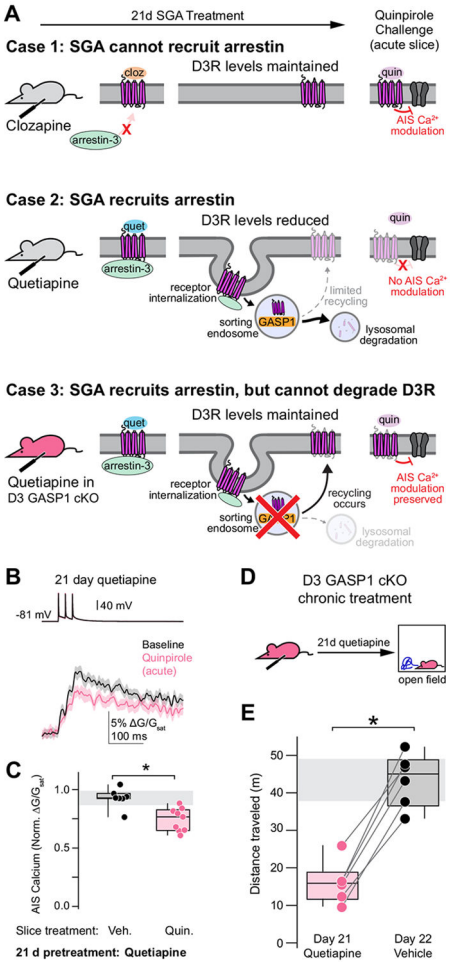

Figure 7.

Quetiapine-induced loss of D3R-$Ca_V3.2$ modulation and development of locomotor tolerance are both mediated by postendocytic sorting of D3R by GASP1. **(A)** Hypotheses for arrestin-dependent modulation of AIS Ca^{2+} following chronic SGA administration. Clozapine, which does not recruit arrestin-3, does not promote D3R endocytosis, preserving D3R-$Ca_V3.2$ modulation (case 1). Quetiapine, which does recruit arrestin-3, promotes D3R endocytosis and degradation, impairing subsequent D3R-$Ca_V3.2$ modulation (case 2). Quetiapine administration in D3 GASP1 cKO eliminates lysosomal degradation and is predicted to preserve D3R-$Ca_V3.2$ modulation (case 3). **(B)** Representative example showing effect of quinpirole on AIS calcium transients in a D3 GASP1 cKO mouse treated with quetiapine. Data shown averaged across 20 trials per condition. Linescan data displayed as mean ± SEM. Baseline, black; quinpirole, pink. **(C)** Summary data comparing the vehicle or quinpirole effect on AIS calcium in D3 GASP1 cKO mice treated for 21 days with either vehicle or quetiapine. Gray bar represents 95% CI of control data. 21-day quetiapine treatment, vehicle slice treatment: 92.8% of baseline, IQR 92.3%–96.7%, $n = 7$ cells from 3 mice; 21-day quetiapine treatment, quinpirole slice treatment: 76.6% of baseline, IQR 65.0%–82.8%, $n = 9$ cells from 3 mice. $p = .005$, Mann-Whitney test. **(D)** D3 GASP1 cKO mice were treated with quetiapine for 21 days. On day 21, they were placed in the

locomotor box immediately following drug injection, and distance traveled was recorded for 60 minutes. The following day, they were injected with vehicle and distance traveled was recorded for 60 minutes. **(E)** Total distance traveled for D3 GASP1 cKO mice treated with vehicle or quetiapine. Gray bar represents 95% CI of control data. Vehicle: 45.0 m, IQR 36.5–48.8 m, $n = 6$ mice; quetiapine: 15.9 m, IQR 11.6–18.8 m, $n = 6$ mice. $p = .016$, Wilcoxon signed-rank test. AIS, axon initial segment; cKO, conditional knockout; D3R, D_3 receptor; IQR, interquartile range; Quin., quinpirole; SGA, second-generation antipsychotic; Veh., vehicle.