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Research Article

RESEARCH ARTICLE

Agonist and Antagonist Effects of Aripiprazole on D_2-Like Receptors Controlling Rat Brain Dopamine Synthesis Depend on the Dopaminergic Tone

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Abstract

Background: The atypical antipsychotic drug aripiprazole binds with high affinity to a number of G protein coupled receptors, including dopamine D_2 receptors, where its degree of efficacy as a partial agonist remains controversial.
Methods: We examined the properties of aripiprazole at D_2-like autoreceptors by monitoring the changes of dopamine synthesis in adult rat brain striatal minces incubated *ex vivo*. The effects of the dopaminergic tone on the properties of aripiprazole were assayed by comparing a basal condition (2 mM K^+, low dopaminergic tone) and a stimulated condition (15 mM K^+, where dopamine release mimics a relatively higher dopaminergic tone). We also used 2 reference compounds: quinpirole showed a clear agonistic activity and preclamol (S-(-)-PPP) showed partial agonism under both basal and stimulated conditions.
Results: Aripiprazole under the basal condition acted as an agonist at D_2-like autoreceptors and fully activated them at about 10 nM, inhibiting dopamine synthesis similarly to quinpirole. Higher concentrations of aripiprazole had effects not restricted to D_2-like autoreceptor activation. Under the stimulated (15 mM K^+) condition, nanomolar concentrations of aripiprazole failed to decrease dopamine synthesis but could totally block the effect of quinpirole.
Conclusions: Under high dopaminergic tone, aripiprazole acts as a D_2-like autoreceptor antagonist rather than as an agonist. These data show that, *ex vivo*, alteration of dopaminergic tone by depolarization affects the actions of aripiprazole on D_2-like autoreceptors. Such unusual effects were not seen with the typical partial agonist preclamol and are consistent with the hypothesis that aripiprazole is a functionally selective D_2R ligand.

Keywords: schizophrenia, striatum, intrinsic efficacy, intrinsic activity, GPCR

Introduction

Aripiprazole (ARI; OPC-14597), a derivative of a dopamine (DA) autoreceptor agonist OPC-4392, is considered as an effective antipsychotic drug with a controversial mechanism of action (Mailman, 2007; Mailman and Murphy, 2010). Compared with other antipsychotics, ARI has a safe and tolerable side-effect profile that includes low potential for extrapyramidal symptoms, weight gain, prolactin elevation, and sedation (Leucht et al., 2009). Because of its clinical efficacy and little chance to provoke adverse events during the therapeutic process, a lot of research has been addressed to explain the unique properties of ARI as well as to

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supply a solid basis for developing a new generation of antipsychotics. According to these observations, ARI seems to be a DA D_2 receptor partial agonist with high affinity binding to many G protein coupled receptors, such as serotonergic, adrenergic, and histaminergic receptors (Shapiro et al., 2003). It is believed that the partial agonism of ARI at D_2 receptor would be the main explanation for its unique clinical profile. Nevertheless, partial agonism at serotonin (5-HT) receptors 5-HT_{1A} and 5-HT_{2C} and antagonism at 5-HT_{2A} may contribute to its special characteristics (Lawler et al., 1999; Shapiro et al., 2003; Bortolozzi et al., 2007). In addition, increasing evidence makes it difficult to consider ARI as a simple D_2 partial agonist. Thus, the functional action of ARI at DA D_2 receptors may vary among agonist, partial agonist, or antagonist depending upon the assessment system, the cell type selected, the function examined, the receptor density, the receptor reserve levels (Shapiro et al., 2003, Tadori et al., 2009), D_2-D_3 heteromerization (Maggio and Millan, 2009), or the surrounding environment, which likely differs between brain regions (Koener et al., 2012). The concept of "functional selectivity" that was proposed by Lawler et al. (1999) and well addressed by Urban et al. (2007a) to interpret the unique behavior of ARI at D_2 receptor seems more appropriate than simply considering it as a partial agonist.

Kikuchi et al. (1995) demonstrated the apparent inconsistency of the behavior of ARI at D_2 presynaptic autoreceptors and D_2 postsynaptic receptors, as it seems to perform as an agonist at the former while as an antagonist at the latter. The antagonism at postsynaptic D_2 receptors has been accepted as a way of controlling symptoms of schizophrenia, based on the therapeutic effect of conventional antipsychotics in treating positive symptoms (Mailman and Murphy, 2010). Additionally, activation of D_2 autoreceptors might also be beneficial for the treatment of some symptoms of schizophrenia (Tamminga, 2002), although this agonistic effect of ARI is controversial, as it appears to be quite dependent upon the surrounding conditions like receptor density or receptor reserve (Tadori et al., 2011a, 2011b). Most of these reports were done in cell lines transfected with a high density of D_{2S} receptor, which is the main subtype of the D_2 autoreceptors (Usiello et al., 2000). Also, the dopaminergic tone in the surrounding milieu has proved to be very important for evidencing the agonistic profile of ARI (Iñiguez et al., 2008). The functional actions of ARI at cloned human D_2-DA receptors show a range of actions (agonism, partial agonism, antagonism) depending upon the cell type and function examined (Lawler et al., 1999; Shapiro et al., 2003). Thus, more reliable evidence from noncloned, native receptor systems is needed to shed light on ARI actions *in vivo*.

In our laboratory, we have developed a method that allows measuring the DA synthesized in dopaminergic neuron terminals in a relatively short period of time (Gonzalez-Sepulveda et al., 2013). Because this is a presynaptic response, it is regulated by the D_2-like autoreceptors located on dopaminergic terminals. Thus, the stimulation or blockade of D_2 autoreceptors will be reflected as changes in DA synthesis. By using this method in the present study, we monitored the direct functional effect of ARI at D_2-like autoreceptors under a low or relatively higher dopaminergic tone elicited by modifying K^+ concentrations.

Materials and Methods

Materials

ARI was kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). (-)-Quinpirole hydrochloride (QUIN) and (*S*)-(-)-sulpiride were from Tocris Bioscience (Minneapolis, MN). Opti-Phase Hisafe-3 liquid scintillation cocktail and [3, 5 - ^{3}H]-L-tyrosine (40–60 Ci/mmol) were supplied by PerkinElmer Wallac (Turku, Finland). ARI and sulpiride were dissolved in ethanol at 10 mM and diluted with water and QUIN was dissolved in water. [3, 5- ^{3}H]-L-tyrosine was purified by high-performance liquid chromatography (HPLC) before use. Preclamol (S-(-)-PPP), ethylenediaminetetraacetic acid (EDTA), HPLC standards and other reagents were purchased from Sigma/RBI (Steinheim, Germany).

[^{3}H]-DA Synthesis

Protocols for animal handling were previously approved by the Ethics Committee for Human and Animal Research (Universitat Autònoma de Barcelona) in accordance with the European Communities Council Directive of 24 November 1986(86/609/EEC). The procedure used was previously described (Gonzalez-Sepulveda et al., 2013). Male Sprague-Dawley rats of 200 to 250 g (Servei d'Estabulari, Universitat Autonoma de Barcelona, Spain) were killed by decapitation between 10:00 and 11:00 AM. Brains were taken out immediately and chilled in modified Krebs-Ringer-bicarbonate medium with the following composition: 120 mM NaCl, 0.8 mM KCl, 2.6 mM $CaCl_2$, 0.67 mM $MgSO_4$, 1.2 mM KH_2PO_4, 27.5 mM $NaHCO_3$, and 10 mM glucose, pH 7.4, saturated with 95% O_2 / 5% CO_2. In a 4°C room, striata (including nucleus accumbens) were dissected and sliced using a McIlwain tissue chopper to obtain minces with a shape that approximates cubes of 0.3 × 0.3 mm/side. Minces were suspended in the same medium and washed by centrifugation and resuspension to remove cell debris. Tissue minces were distributed into 2-mL polypropylene tubes and preincubated for 2 hours at 37°C in an Eppendorf Thermomixer under 95% O_2 / 5% CO_2 atmosphere. ARI, QUIN, S-(-)-PPP, or vehicle were added at the latest 10 minutes of preincubation. Then ring-labeled [3, 5- ^{3}H]-L-tyrosine (40–60 Ci/mmol) was added to all samples (final concentration of 0.12 µM), and incubation continued for 10 minutes more to synthesize [^{3}H]-DA. A depolarizing condition was obtained by increasing the K^+ in the Krebs buffer by adding concentrated KCl. This depolarizing medium was added 10 minutes before ARI or QUIN. When the D_2 antagonist sulpiride was used, it was added 10 minutes before ARI or QUIN. The synthesis was stopped by the addition of a deproteinizing solution containing trichloroacetic acid and 100 nmol internal standard DA per tube. Blank tubes contained deproteinizing solution prior to addition of [^{3}H]-tyrosine and were kept ice-cold throughout. All samples were homogenized in a Dynatech/Sonic Dismembrator (Dynatech Labs, Chantilly, VA). An aliquot was taken for protein quantification by the Lowry method to take into account the variability of tissue amounts in each tube. Tissue homogenates were then centrifuged (12,000 *g*, 10 minutes, 4°C), and supernatants were recovered for [^{3}H]-DA purification by HPLC-UV.

The chromatographic system consisted of a reverse-phase C_{18} column (Tracer Extrasil ODS2, 5-µm particle size, 25 x 0.46 cm; Teknokroma, Spain) and an ion-pair mobile phase made of 100 mM sodium phosphate buffer, 1 mM EDTA, and 0.75 mM octanesulfonic acid plus 12 % (vol/vol) methanol at pH 5. Flow rate was 1 mL/min. Internal standards were detected by UV 285 nm. Radiolabelled and endogenous tyrosine and DA were undetectable by UV absorbance. Recovery of the internal standard was quantified in each sample (internal/external standard peak area). DA fractions were collected in scintillation vials, mixed with Optiphase HiSafe III cocktail, and [^{3}H]-DA was quantified in a liquid scintillation counter. Disintegrations per minute (dpm) obtained were corrected by DA internal standard recovery, dpm in blank samples, and protein content in each incubated

tube. Results were expressed as percentage vs control samples in the same experiment. Concentration-response curves were obtained after normalization to percent of controls in the same experiment, pooling of data, and nonlinear fit to sigmoidal dose response. The concentration curve of ARI in 2 mM K^+ was best fitted to a 2-site competition curve.

Results

10 nM ARI Inhibits DA Synthesis through D_2-Like Receptors under 2 mM K^+ (Basal Conditions)

Activation of D_2 receptors can inhibit DA synthesis in dopaminergic neurons in rat brain striatum. To confirm this, the well-known D_2 receptor agonist quinpirole (QUIN) was used as a standard reference. Under 2 mM K^+ (basal condition), QUIN decreased DA synthesis in a dose-dependent manner. As shown in Figure 1a, QUIN effects followed a 1-site model with the following parameters: Emax = 47.8 ± 0.42%; EC50 = 11.4 nM. This effect was totally blocked by the selective D_2 receptor antagonist sulpiride (data not shown).

Under the same experimental conditions, we obtained the concentration-response curve of ARI on the inhibition of DA synthesis shown in Figure 1b. Unlike the dose-response curve of QUIN, this curve best fitted with a 2-site model with the following parameters: Emax = 69 ± 0.43%, $EC50_1$ = 0.93 nM, and $EC50_2$ = 0.21 µM (Table 1). Compared with the QUIN dose curve, we made the hypothesis that only the first part of the ARI dose-response curve was due to its effects on D_2 autoreceptors. To test this hypothesis, the typical selective D_2 receptor antagonist sulpiride was applied to block D_2 receptors. Sulpiride at 1 µM fully eliminated the inhibitory effect of ARI at 10 nM on DA synthesis, which was consistent with our hypothesis (Figure 2a). In addition, QUIN failed to make a concentration-related response when added to the incubations 10 minutes after ARI 10 nM, as shown in Figure 2b. The comparisons between the 2 dose-response curves of QUIN (one in the absence and the other in the presence of ARI 10 nM; Figure 2b) indicate that the D_2 autoreceptors have already been activated by ARI 10 nM. These data strengthen our hypothesis that low doses (≤100 nM) of ARI inhibit DA synthesis due to its action on D_2-like receptors. To further address this issue, we examined whether the effects of a higher concentration of ARI (1 µM) could be blocked by sulpiride. Sulpiride (1 µM) partially blocked the effect of 1 µM ARI (from −50% to −32%; Figure 2c), but could not eliminate it completely. Thus, it is likely that micromolar concentrations of ARI inhibit DA synthesis through D_2 and non-D_2 mediated mechanisms.

ARI Failed to Activate D_2 Autoreceptors under 15 mM K^+ (Stimulated Conditions)

Increasing the potassium concentration in Krebs buffer from 2 to 15 mM increased the amount of DA released, as expected. In preliminary experiments, the percent of newly synthesized [^{3}H]-DA released to the medium under 2 mM K^+ was 1.13 ± 0.95% (mean ± SD, N = 23 incubations) while under 15 mM K^+ was 7.5 ± 3.0% (N = 38 incubations). This led to a 6.6-fold increase in DA released. The increased concentrations of extracellular DA may facilitate its interaction with D_2 autoreceptors. Previous reports have shown that the dopaminergic tone played a very important role in evidencing the agonist property of ARI (Iñiguez et al., 2008). Similarly, in our present study, we also observed different effects of ARI under different extracellular DA concentrations, which could also represent differences in the dopaminergic

Table 1. Parameters of Quinpirole, Aripiprazole, and S-(-)-PPP Inhibition of Rat Brain [^{3}H]-Dopamine Synthesis under Basal (2 mM K^+) or Stimulated (15 mM K^+) Conditions Representing Low- and High-Dopaminergic Tone, Respectively

	2 mM K^+	15 mM K^+
Quinpirole		
IC_{50}, nM	11	9.3
Emax, %	49	39
Aripiprazole		
IC_{50} (1st/2nd component)	0.93 nM / 0.21 µM	68 nM
Emax (1st / 2nd component), %	41/69	17
Preclamol (S-(-)-PPP)		
IC_{50}, nM	0.11	0.051
Emax, %	34	31

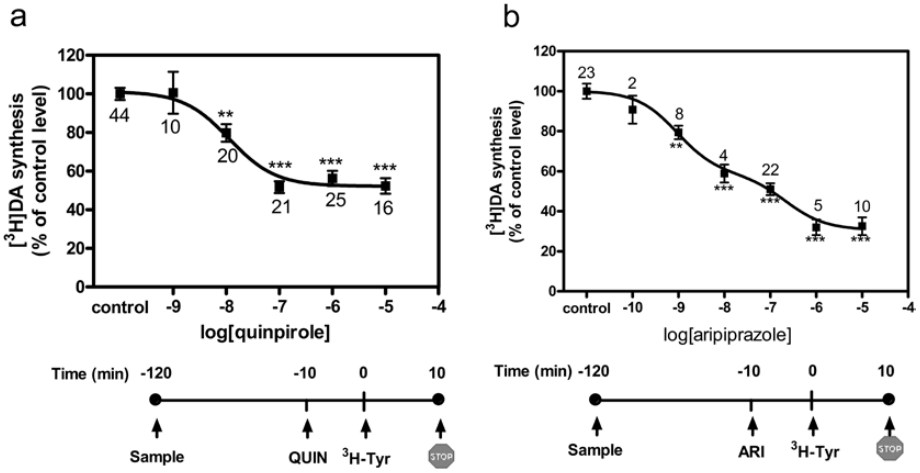

Figure 1. Concentration-response curve of quinpirole (QUIN) (a) and aripiprazole (ARI) (b) on the inhibition of dopamine (DA) synthesis in nonstimulated rat brain striatal minces (basal condition: 2 mM K^+). Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated over the symbols. *P < .05, **P < .01, and ***P < .001 vs control (1-way ANOVA followed by Bonferroni's test). Data were fitted to a sigmoidal dose-response (a) or a 2-site competition (b) curve. Parameters obtained are listed in Table 1.

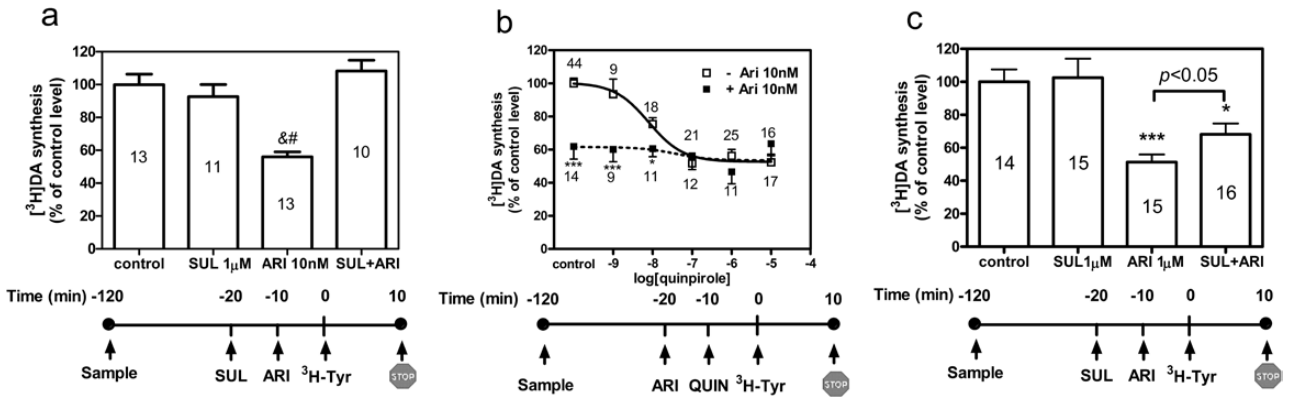

Figure 2. Inhibition of dopamine (DA) synthesis by 10 nM aripiprazole (ARI) is mediated by D_2 receptors. a, Striatal minces were incubated with sulpiride (1 µM) and ARI (10 nM) either alone or together under a 2-mM K^+ condition. Sulpiride 1 µM could totally eliminate the effect of ARI at 10 nM. b, Concentration-response curve of quinpirole (QUIN) in the presence of 10 nM ARI under a 2-mM K^+ condition. C, Sulpiride at 1 µM only partially blocks the inhibitory effect of ARI at 1 µM. Striatal minces were incubated under 2 mM K^+. Experimental design is shown in a time bar under each graph. Data represent the means ± SEM of N incubations indicated by the symbols or inside bars. $^{\&}P<.001$ vs control, $^{\#}P<.001$ vs group treated with both ARI 10 nM and sulpiride 1 µM; $^{*}P<.05$ and $^{***}P<.001$ vs group treated in the absence of ARI at the same dose. c, Statistical difference between both ARI-treated groups is expressed by a connecting line (1-way ANOVA followed by Bonferroni's test).

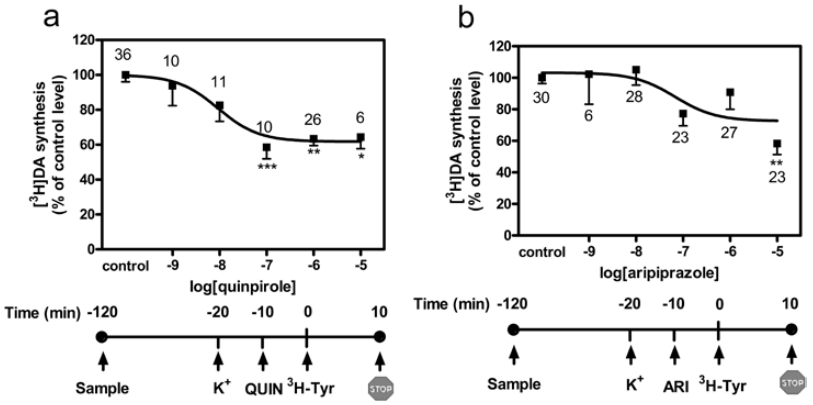

Figure 3. Concentration-response curve of quinpirole (QUIN) (a) and aripiprazole (ARI) (b) on the inhibition of dopamine (DA) synthesis in stimulated rat brain striatal minces (high-dopaminergic tone condition: 15 mM K^+). Experimental design is shown in the time bar under each graph. Data represent the means ± SEM of N incubations indicated by the symbols. $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$ vs control (1-way ANOVA followed by Bonferroni's test). Data were fitted to a sigmoidal dose-response curve. Parameters obtained are listed in Table 1.

tone (Table 1; Figure 3). Under 15 mM potassium conditions, ARI failed to inhibit DA synthesis at doses <10 µM (Figure 3b). We also measured the effect of QUIN under the same situation. As a typical D_2 agonist, QUIN acted as a stable agonist under both conditions, as the dose-response curve of QUIN under 15 mM K^+ was quite similar with the curve under 2 mM K^+ (Figures 1a and 3a), and the parameters for the 2 curves did not differ much either (Table 1). The reason why ARI failed to stimulate D_2 autoreceptors in the depolarizing conditions could be either because ARI failed to bind to D_2 receptors in the presence of the high concentration of DA or because ARI managed to bind but failed to stimulate D_2 receptors more efficiently than DA, behaving like an antagonist. To determine the reason, we treated the brain minces with both ARI and QUIN and observed that 10 nM ARI could totally eliminate the effects of QUIN at either 10 or 100 nM (Figure 4). These results prove that ARI binds D_2 receptors under these conditions but behaves as an antagonist under high dopaminergic tone. This result is fully consistent with a high affinity and low efficacy of ARI for the stimulation of D_2 receptors. Moreover, in the presence of 15 mM K^+, 10 nM ARI tended to increase DA synthesis (Figure 4), as it would be expected for an antagonist of K^+-released DA. To further characterize the influence of K^+ in the properties of ARI, we gradually increased K^+ concentrations from 2 to 15 mM K^+ to increase the probability of DA release and tested the agonism elicited by 100 nM ARI. We found that under a 8-mM K^+ threshold, ARI had agonist effects (Figure 5). These effects appeared full or partial depending on K^+ concentrations and were clearly lost at higher K^+. It seems likely that a higher extracellular DA concentration rendered ARI unable to stimulate D_2 receptors more efficiently than the endogenous neurotransmitter, although alternative effects of depolarization on membrane environment cannot be ruled out (Sahlholm et al., 2011).

Comparison of the Effects of ARI with the Partial Agonist Preclamol (S-(-)-PPP)

Preclamol (S-(-)-PPP) has been extensively characterized as a D_2 receptor partial agonist (Clark et al., 1985; Lathi et al., 1998; Tadori et al., 2005). To assess whether the effects of ARI were unique to this compound or shared with other D_2 partial agonists, we assayed the effects of S-(-)-PPP on DA synthesis under

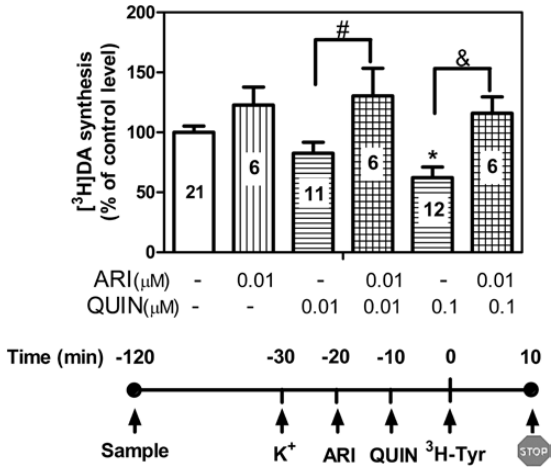

Figure 4. 10 nM aripiprazole (ARI) blocks quinpirole (QUIN) inhibition of dopamine (DA) synthesis under 15-mM K^+ conditions. Minces were treated with ARI (10 nM) and QUIN (10 and 100 nM) either alone or together. Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated inside the bars. $^{*}P < .05$ vs blank control. $^{\#}P < .05$ and $^{\&}P < .05$ in the 2 groups of connected bars (1-way ANOVA followed by Bonferroni's test).

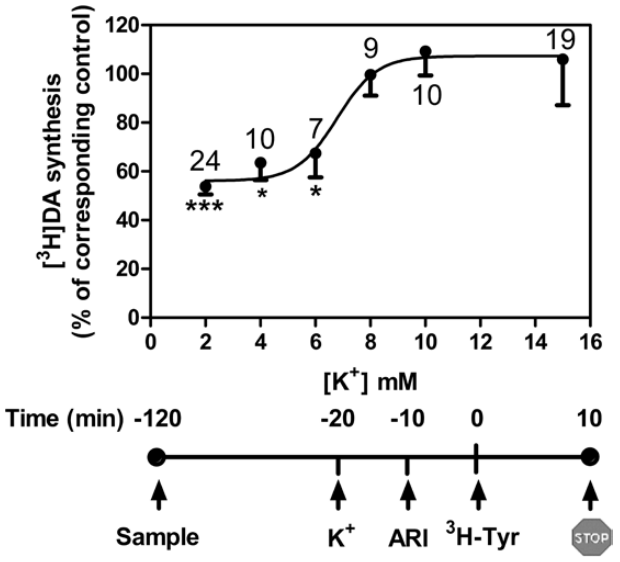

Figure 5. Titration of aripiprazole (ARI) effects on dopamine (DA) synthesis vs increasing K^+ concentrations. ARI (100 nM) behaved as an agonist with K^+ concentrations up to 6 mM in our brain minces incubations. Experimental design is shown in the time bar under the graph. Data shown represent means ± SEM of N incubations indicated over the symbols, normalized to the respective control group under the same K^+ concentration (not show in the graph). $^{*}P < .05$ and $^{***}P < .001$ vs control group under the same K^+ concentration (1-way ANOVA followed by Bonferroni's test).

2 and 15 mM K^+ conditions. We found that S-(-)-PPP did inhibit DA synthesis quite similarly in both situations (Figure 6a and b), with maximal effects (–34 and –31%, respectively) smaller than those of ARI or QUIN. Although such small maximal effects are consistent with literature data on S-(-)-PPP, the shape of the concentration response curve appeared biphasic, with significant decreases of DA synthesis at 1 nM and 1 μM and some nonsignificant points at the high nanomolar range and 10 μM as well (Figure 6). As the reported affinity of S-(-)-PPP for D_2 receptors is in the high-nanomolar/low-micromolar range (Clark et al., 1985; Burris et al., 2002; Tadori et al., 2005), we were curious about the 1 nM S-(-)-PPP significant effects. We found that the decrease in DA synthesis elicited by 1 nM S-(-)-PPP was completely reversed by 100 nM sulpiride (Figure 6c). Thus, it seems likely that S-(-)-PPP elicits dual effects on DA synthesis quite different from those previously observed with ARI.

Discussion

In the present paper, we show that ARI actions on native D_2-like autoreceptors in rat brain presynaptic dopaminergic terminals can be those of an agonist or an antagonist, depending on whether the experimental conditions mimic low or high dopaminergic tone. These results are consistent with the well-known low activity of ARI to stimulate D_2 receptors that is characteristic of a partial agonist when compared with DA. However, we must note that important differences were found between ARI and preclamol (S-(-)-PPP), another well-characterized D_2 receptor partial agonist that seems to work differently. The strength of these results is that we use an approach that can be considered more physiologically relevant than previous articles in receptor-transfected cells. First, D_2 receptors involved were neuronal. Second, DA competing with ARI was released by K^+ stimulation of brain tissue *ex vivo*. And finally, the response measured is [^{3}H]-DA synthesis, a specific process of catecholaminergic neurons, mostly dopaminergic in the striatum. However, these strengths bring together the challenge of the correct interpretation of results obtained in this complex tissue sample, where other receptors and brain terminals and other cells are also naturally present.

Presynaptic D_2 receptors are thought to be the D_{2S} subtype compared with the full-length D_{2L} subtype (Usiello et al., 2000). Previous work by Kikuchi et al. (1995) suggested that the agonist or antagonist properties of ARI were dependent on the presynaptic vs postsynaptic localization of D_2 receptors, respectively. However, here we show that ARI can show both agonist and antagonist properties at the same presynaptic location controlling DA synthesis. This indicates that the subtype of D_2 receptor involved does not explain the switch in the behavior of ARI. In agreement, later work by Kikuchi's group (Tadori et al., 2005, 2009, 2011a, 2011b) found that ARI can be agonist and antagonist on recombinant human D_{2S} receptors, depending on the level of receptor expression and competing DA. Thus, our results validate in brain tissue the later hypothesis of these authors. Presynaptic D_2-like receptors can be fully stimulated by ARI provided that competing DA is maintained at low levels. In contrast, during nerve terminal release of DA, ARI behaves as an antagonist because of its lower efficacy to stabilize the active conformation of D_2 receptors compared with DA. The subtype of D_2-like receptors controlling DA synthesis likely is the D_{2S}, although a minor contribution of D_3 receptors or postsynaptic D_2 receptors cannot be completely ruled out (Usiello et al., 2000; Anzalone et al., 2012).

Previous work also showed that the intrinsic activity of D_2 partial agonists seems to depend on receptor reserve (Meller et al., 1987; Burris et al., 2002). A partial agonist like ARI might stabilize D_2 receptors in a conformation that activates G proteins only for short periods or with low efficacy, leading to higher dissociation constants of the receptor-G protein complex. As a consequence, it may need to occupy significantly more receptors than the full agonist QUIN to reach a similar maximal agonist effect. These "extra" receptors available for the agonist properties of ARI can come from the so-called receptor reserve (Figure 7). A higher receptor reserve has been

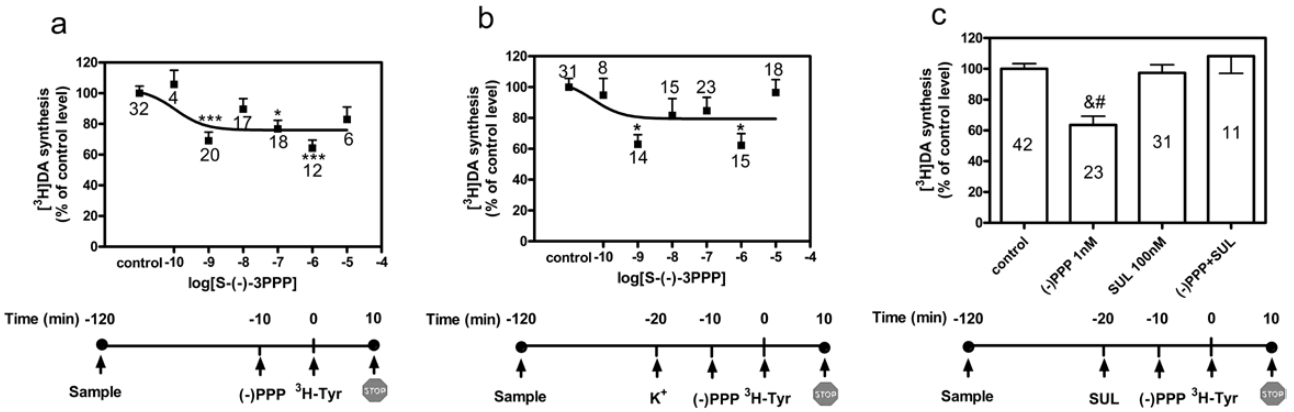

Figure 6. The effects of the D_2 partial agonist preclamol (S-(-)-PPP) differ from those of aripiprazole (ARI) previously shown. Concentration-response curves of S-(-)-PPP on the inhibition of dopamine (DA) synthesis in (a) nonstimulated (basal condition: 2 mM K^+) or (b) stimulated rat brain striatal minces (high-dopaminergic tone condition: 15 mM K^+). c, The effect of 1 nM S-(-)-PPP is completely blocked by sulpiride (100 nM). Experimental design is shown in the time bar under the graph. Data represent the means ± SEM of N incubations indicated over the symbols inside bars. $^*P<.05$ and $^{***}P<.001$ vs control. $^{\&}P<.001$ vs control, $^{\#}P<.001$ vs group treated with both S-(-)-PPP 1 nM and sulpiride 100 nM (1-way ANOVA followed by Bonferroni's test). Data were fitted to a sigmoidal dose-response curve. Parameters obtained are listed in Table 1.

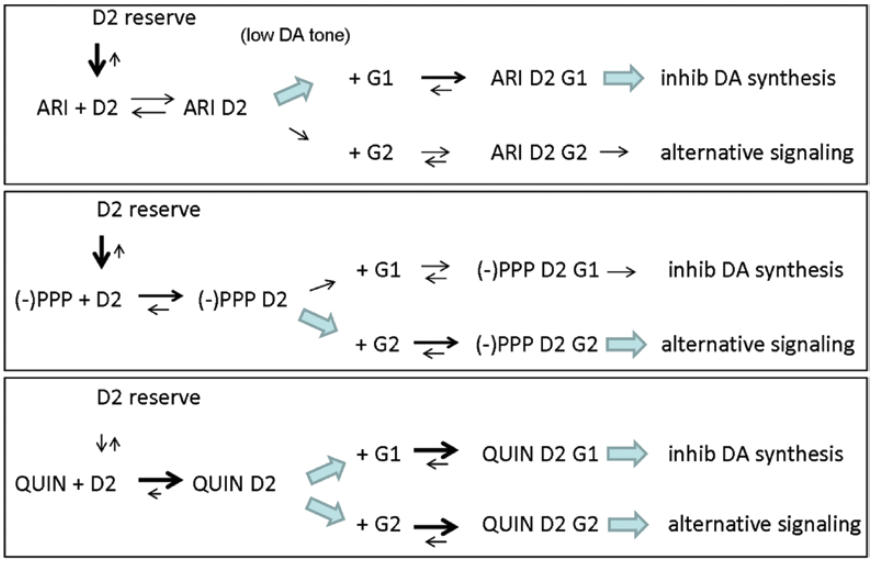

Figure 7. Hypothetical model explaining the actions of partial agonists aripiprazole (ARI) and preclamol ((-)PPP) and the full agonist quinpirole (QUIN) on dopamine (DA) synthesis. Both partial agonists would mobilize the receptor reserve, as a difference with the full agonist quinpirole. The 2 partial agonists would, however, stabilize different conformations of the D_2 receptors, leading to functional selectivity for signaling proteins. G1 and G2 could be different G proteins or other signaling molecules competing for binding to the active receptor. ARI would stimulate G1 only if a low-dopaminergic tone is present, since DA more efficiently activates D_2 receptors than ARI.

hypothesized for presynaptic D_2 receptors compared with postsynaptic D_2 receptors (Meller et al., 1987). Therefore, a partial agonist like ARI will appear to have higher intrinsic activity at presynaptic D_2 receptors vs postsynaptic D_2 receptors due to receptor reserve. This assumption fits experimental data (Kikuchi et al., 1995; Burris et al., 2002). Nevertheless, a high receptor reserve is necessary but not sufficient for ARI to show its agonist properties: a low dopaminergic tone is also required. Previous papers showing agonist effects of ARI on DA synthesis required a low dopaminergic tone, achieved by reserpine or gamma-butyrolactone treatment to animals (Kikuchi et al., 1995; Iñiguez et al., 2008). Reserpine depletes DA stores, and gamma-butyrolactone is considered a nerve impulse inhibitor preventing DA release. In our work, an interruption of nerve impulses takes place at slicing striatal tissue by cutting off nerve endings from dopaminergic cells bodies. Therefore, our basal conditions (2 mM K^+) may well be considered relatively similar to gamma-butyrolactone in that they produce a low dopaminergic tone, where only 1% of newly synthesized [^{3}H]-DA is released to the medium. Accordingly, we may wonder whether ARI is actually able to act as an agonist *in vivo*, where dopaminergic tone might be fluctuating within a range difficult to define. Indeed, human results suggest that ARI has actions consistent with agonism on D_2 receptors controlling prolactin release, in contrast with most antipsychotics (Safer et al., 2013). Whether ARI acts as a D_2 agonist on nerve terminals involved in psychosis remains speculative, but it is worth considering, as it led to its discovery (Kikuchi et al., 1995). Beneficial effects of selective dopaminergic autoreceptor agonists have been postulated for the treatment of psychosis (Tamminga, 2002).

However, if a high dopaminergic tone is expected during psychosis, the antagonistic action of ARI on both pre- and postsynaptic D_2 receptors will more likely prevail and correlate with its antipsychotic effects. On the other hand, the postulated "DA stabilization" elicited by ARI would only make sense on presynaptic D_2 receptors, which have sufficient receptor reserve to account for ARI agonist and antagonist properties. The lower receptor reserve of postsynaptic D_2 receptors would only allow antagonist-like effects of ARI, based on the lack of sufficient G protein activation. Therefore, postsynaptic "DA stabilization" would not be possible unless alterations in receptor sensitivity or functional selectivity add new levels of complexity to the postsynaptic actions of ARI (Meller et al., 1987; Mailman, 2007; Mailman and Murphy, 2010).

The difference in maximal effects between ARI and S-(-)-PPP cannot be explained solely by the receptor reserve hypothesis. S-(-)-PPP usually shows higher intrinsic activity than ARI under several assays (Burris et al., 2002; Tadori et al., 2005; Kehne et al., 2008), so we were surprised by the lower maximal effect of S-(-)-PPP compared with ARI (–34% vs –41%, respectively, at 2 mM K^+ (Figure 6a; Table 1). Similarly, literature binding data show that the conformation of D_2 receptors stabilized by ARI might be closer to that of a typical antagonist compared with that stabilized by S-(-)-PPP (Burris at al., 2002; Urban et al., 2007b; but see also Shapiro et al., 2003). Intrinsic activity of D_2 receptor partial agonists depends on the assay used (Kehne et al., 2008), which could be due to functional selectivity vs several intracellular cascades and their respective amplification mechanisms (Kenakin and Morgan, 1989). The fact that in our functional assay ARI was more efficient than S-(-)-PPP may be an indication of preferred functional selectivity of ARI vs the transduction pathway inhibiting DA synthesis. In fact, Mottola et al. (2002) found evidence of D_2 agonists having the opposite functional selectivity, with poor effects on D_2-controlled DA synthesis, which gives support to the functional selectivity hypothesis. In addition, we were also surprised by the shape of the concentration curve S-(-)-PPP, which suggests a complex interaction with receptors controlling DA synthesis. The rise of extracellular DA elicited by 15 mM K^+ does not seem to affect it, which is similar to QUIN, but not to ARI. Although a detailed study of S-(-)-PPP effects exceeds the scope of this paper, its effects seem to be D_2 mediated (Figure 6c; but see Largent et al., 1987). Alternative explanations can be considered, such as better oligomerization of some receptor conformations than others, although at present this would be highly speculative (Navarro et al., 2013).

When DA release was stimulated by 15 mM K^+, nanomolar concentrations of ARI did not decrease [^{3}H]-DA synthesis like QUIN or S-(-)-PPP, but instead they clearly blocked QUIN action, behaving as an antagonist. In addition, in the absence of QUIN, we observed a slight tendency to increase [^{3}H]-DA synthesis in samples treated with ARI. This could reveal ARI antagonism of K^+-released endogenous DA stimulating D_2 receptors. We do not know how much of endogenous DA was released by 15 mM K^+ or the concentrations of extracellular DA achieved, because these values would be hard to obtain within the same experiment in our conditions. We know from preliminary experiments that the percent of newly synthesized [^{3}H]-DA released by 15 mM K^+ increases by 6.6-fold on average compared with 2 mM K^+. We hypothesized that this K^+ stimulation would model a higher dopaminergic tone in rat brain minces *ex vivo*, but we must acknowledge that membrane depolarization may also have additional effects on D_2 receptor conformation and interactions with signaling molecules (Sahlholm et al., 2011). Furthermore, any comparison of our results with dopaminergic tone in human schizophrenics would be speculative. Nevertheless, we observed a remarkable switch of ARI behavior from agonist to antagonist by increasing K^+. Therefore, our method could be useful for future pharmacological research of newer $D_{2/3}$-based antipsychotics with low liability of side effects based on clinical experience with ARI. New compounds like brexpiprazole or cariprazine are being clinically tested. Additional D_2 partial agonists already exist, but ARI has been the most clinically successful so far. An important question to consider on this issue is the degree of intrinsic efficacy of a partial agonist that is optimal for the best antipsychotic effects (Tamminga, 2002). ARI is considered to have low intrinsic efficacy; that is, its activity is closer to that of an antagonist than to that of an agonist. Previous D_2 partial agonists tested may have failed to display the adequate intrinsic activity for antipsychotic clinical effects (Lathi et al., 1998; Tadori et al., 2005; Natesan et al., 2010). An important additional question is whether there is a clear relationship between D_2 partial agonism and low liability of extrapyramidal side effects. Two main hypotheses are currently being considered. First, D_2 occupancy by antagonists might have a threshold over which extrapyramidal effects would appear. And second, transient D_2 blockade would elicit less extrapyramidal side effects than continuous blockade (Seeman, 2002). Surprisingly, ARI occupancy of D_2 receptors at clinically relevant doses has been shown to be well above the threshold considered for other antipsychotics, and ARI is not relatively fast at dissociating from D_{2L} receptors (Kuroki et al., 2002; Seeman, 2005; Maggio and Millan, 2010). This suggests that ARI action on D_2 conformation might contribute to low liability of extrapyramidal effects. The low efficacy of ARI - D_2 - G protein activation might not elicit significant postsynaptic responses, but it may allow a higher D_2 receptor occupancy than other antipsychotics without producing extrapyramidal side effects.

Our determination of [^{3}H]-DA synthesis in brain tissue *ex vivo* is methodologically faster than classical DOPA accumulation *in vivo*. We obtained 24 incubations of brain minces from the striata of a single rat. QUIN and ARI IC_{50} values obtained were very similar to those reported in the literature (Table 1). QUIN reached its maximal effect (48% decrease) at 100 nM. In contrast, the maximal effect of ARI was a 68% decrease at the concentration 1 μM, with a curve best adjusted to 2 sites. ARI not only has affinity for D_2 receptors, it also has significant affinity for a large number of other G-protein coupled receptors, such as serotonergic, adrenergic, and histaminergic receptors (Shapiro et al., 2003). The low-affinity effect of ARI on [^{3}H]-DA synthesis could be related to actions on serotonin or other receptors, as it has a higher IC_{50} than D_2-mediated effects (Table 1) and it was not blocked by 1 μM sulpiride (Figure 2c). The selective 5-$HT_{1A/7}$ receptor agonist R(+)-8-OH-DPAT attenuates amphetamine-induced DA synthesis in rat striatum (Kuroki et al., 2000), and also other agonists of 5-HT_{1A} receptors inhibit tyrosine hydroxylase in rat striatum (Johnson et al., 1996). We must note, however, that 5-HT_{1A} receptors are considered as somatodendritic and unlikely present in dopaminergic terminals. The serotonergic actions of ARI deserve further investigation, as they could contribute to its unique antipsychotic effects (Jordan et al., 2002; Shapiro et al., 2003; Bortolozzi et al., 2007). Here in our study, the non-D_2 receptor effect of ARI on the inhibition of DA synthesis might be related with serotonin 5-$HT_{1A/7}$, 5-HT_{2C} receptors, or receptors for additional neurotransmitters (DiMateo et al., 2001; Shapiro et al., 2003), but further studies are needed to confirm these hypotheses. The low presence of serotonergic receptors in dopaminergic terminals open the possibility that the non-D_2 effects of ARI on DA synthesis are indirectly mediated by GABA

or glutamate terminals also present in our minces of striatal tissue. This effect does not disappear under the 15 mM K^+ condition, which also differentiates it from D_2-mediated effects.

In conclusion, we observed different properties of ARI under different levels of dopaminergic tone in the surrounding milieu: agonist under low dopaminergic tone and antagonist under a relatively higher dopaminergic tone elicited by increasing K^+ concentrations. This can be based on the low activity of ARI as agonist and the high receptor reserve of presynaptic D_2 autoreceptors, but the functional selectivity hypothesis must be taken into consideration, as it has different effects than S-(-)-PPP. In contrast, the D_2 receptor full agonist QUIN acted as a clear agonist under both conditions. We also observed an effect of ARI at higher concentrations that is not apparently D_2 mediated. These results indicate that ARI effects on presynaptic D_2-like receptors in brain tissue have unique properties that clearly differentiate it from other traditional antipsychotics.

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Statement of Interest

The authors declare no financial interest or conflict to disclose.

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