Pharmacology, Biochemistry and Behavior 93 (2009) 141–147

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Using prepulse inhibition to detect functional D3 receptor antagonism: Effects of WC10 and WC44

Martin Weber [a], Wei-Li Chang [a], John P. Durbin [c], Paula E. Park [a], Robert R. Luedtke [b], Robert H. Mach [c], Neal R. Swerdlow [a,*]

[a] Department of Psychiatry, UCSD School of Medicine, 9500 Gilman Dr., La Jolla, CA 92037-0804, United States
[b] Department of Pharmacology and Neuroscience, UNT Health Science Center, United States
[c] Malinckrodt Institute of Radiology, WUSTL School of Medicine, United States

ARTICLE INFO

Article history:
Received 31 March 2009
Received in revised form 28 April 2009
Accepted 30 April 2009
Available online 6 May 2009

Keywords:
Dopamine
D3 selective compounds
Prepulse inhibition
Schizophrenia

ABSTRACT

Prepulse inhibition of startle (PPI) is an operational measure of sensorimotor gating that is impaired in schizophrenia. Treatment with mixed dopamine D2/D3 antagonists diminishes schizophrenia symptoms, and opposes dopamine agonist-induced PPI deficits in rats. There are reasons to believe that functional D3 receptor antagonists might offer more favorable therapeutic profiles compared to current antipsychotics. However, D3-related drug discovery is hampered by the absence of assays sensitive to D3-mediated (antipsychotic) properties *in vivo*. Here, we characterized two putative D3-active compounds – WC10 and WC44 – in a PPI-based screening assay, comparing the sensitivity of test compounds to oppose PPI deficits induced by the mixed D1/D2-like agonist apomorphine vs. the preferential D3 agonist pramipexole in rats. WC10, WC44 (0, 1, 3, 10 mg/kg, each), and the preferential D2 antagonist L741,626 (0, 1 mg/kg) were studied, in combination with apomorphine (0, 0.5 mg/kg), or pramipexole (0, 1 mg/kg). L741,626 prevented apomorphine-, but not pramipexole-induced PPI deficits. WC10, but not WC44, prevented apomorphine-induced PPI deficits; both compounds opposed pramipexole-induced PPI deficits, suggesting functional D3 and D1/D2 antagonist profiles for WC10, and functional D3 receptor antagonism for WC44. This assay may be valuable for detecting predominantly D3 vs. D2 receptor-linked mechanisms of action *in vivo*.

Published by Elsevier Inc.

1. Introduction

It is suggested that some of the therapeutic effects of D2/D3 antagonist antipsychotics are mediated via blockade of D3 receptors, while their extrapyramidal side effects are due primarily to D2 receptor antagonism (Sokoloff et al., 1990). One basis for this hypothesis is that, compared to D2 receptors, D3 receptors are localized primarily in limbic and mesolimbic regions, while D2 receptors are distributed throughout the striatum. Very high densities of D3 receptors are found in the nucleus accumbens (NAC) of both rats and primates (c.f. Sokoloff et al., 2006), and this brain region is implicated in both the pathophysiology of schizophrenia and the therapeutic mechanisms of antipsychotics (cf. Gurevich et al., 1997). Preferential antagonists or partial agonists for D3 receptors might thus offer therapeutic advantages over current antipsychotics.

Developing compounds with a D3 preferential *in vivo* profile, however, is complicated by the high sequence homology of D3 and D2 receptors (cf. Luedtke and Mach, 2003). Further, such compounds may be subject to functional selectivity, i.e. may act as agonist, partial agonist, or antagonist on a given receptor, depending on tissue types, availability of certain G-proteins and the intracellular machinery linked to these receptors/G-proteins (c.f. Mailman, 2007). This raises the possibility that DA compounds that have been characterized as agonists or antagonists for specific DA receptor subtypes *in vitro* might nonetheless have different functional properties *in vivo*. Thus, while the D3 receptor is a promising target for antipsychotic development, and *in vitro* studies have been very valuable in identifying compounds that preferentially bind to D3 receptors (c.f. Joyce and Millan, 2005), better models are needed, with 1) predictive validity for antipsychotic-like function; 2) sensitivity for the detection of a predominant D3 related (vs. D1/D2 related) mechanism of action *in vivo*.

One valuable, translational and predictive model for antipsychotic function is prepulse inhibition (PPI). PPI is an operational measure of sensorimotor gating, defined by the reduction in startle magnitude following a weak prestimulus. PPI is impaired in unmedicated schizophrenia patients (Braff et al., 1978; Swerdlow et al., 2006) as well as their unaffected first-degree relatives (Cadenhead et al., 2000; Kumari et al., 2005), and a recent study linked polymorphisms of the DA D3 receptor to levels of PPI in healthy controls (Roussos et al., 2008). In rats, PPI deficits are induced by DA agonists such as the direct D1/D2-like agonist apomorphine (APO) and the indirect DA agonist amphetamine (Swerdlow et al., 1986; Mansbach et al., 1988). PPI is potently regulated by brain regions rich in D3 receptors, in particular the NAC

* Corresponding author. Tel.: +1 619 543 6270; fax: +1 619 543 2493.
E-mail address: nswerdlow@ucsd.edu (N.R. Swerdlow).

0091-3057/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.pbb.2009.04.022

(Swerdlow et al., 1986; c.f. Swerdlow et al., 2008), and D3-preferential agonists such as 7-OH-DPAT, ropinirole and quinelorane disrupt PPI in rats and humans (Caine et al., 1995; Giakoumaki et al., 2007; Swerdlow et al., 1998; Varty and Higgins, 1998). However, the limited D3 vs. D2 binding preference of these compounds makes it difficult to assess the relative contribution of D3 vs. D2 receptor activation to these effects.

This study assessed the ability of the rat PPI model to detect compounds with predominant functional D3 antagonism *in vivo*, based on a *greater sensitivity* to prevent PPI deficits induced by a preferential D3 agonist (e.g. pramipexole (PRA)) compared to a non-selective D1/D2 agonist (e.g. APO). This strategy is based on the contrasting binding affinities of PRA and APO to DA receptor subtypes. Millan et al. (2002) reported that the binding affinity of PRA to the D3 receptor (hD3) was 90-fold higher relative to the short isoform of the receptor (D2S), 160-fold higher relative to the long isoform (D2L), and more than 10,000-fold relative to the D1 receptor (hD1). In contrast, the binding affinity of APO to the hD3 receptor was only 1.35-fold higher than to the D2S, 3-fold higher than to the D2L, and only 14-fold higher than to the hD1 receptor, confirming the non-selectiveness of APO to these DA receptor subtypes. These data indicate that a positive finding with the *in vivo* assay used here could potentially provide functional evidence of greater D3 than D1/D2 selectivity, and predict potentially novel clinical profiles. Another important basis for this *in vivo* assay is the finding that the selective D2 antagonist L741,626 is more sensitive in its ability to prevent PPI deficits caused by APO than those caused by PRA (Weber et al., 2008). This suggests a critical role of D2 receptor activation in the PPI-disruptive effects of APO, but not PRA. A similar approach was used by Zhang et al. (2007) to demonstrate that SB-277011-A and A-69110 – two putative D3 receptor antagonists – prevented PPI deficits induced by the preferential D3 agonist PD128907, but *not* those induced by APO.

WC10 and WC44 are phenylpiperazine derivates (Fig. 1) that have been characterized in forskolin-stimulated adenylate cyclase activity assays *in vitro*, using cell systems expressing either D3- or D2 receptors (Chu et al., 2005). Using these and binding assays, WC10 has been characterized as an antagonist/weak partial agonist with a D3:D2 binding ratio of 43, and WC44 as a full D3 agonist (but see below) with a D3:D2 binding ratio of 23 (Chu et al., 2005). In the present studies, the potential preclinical antipsychotic-like profile of WC44 was evaluated in measures of PPI deficits induced by APO and PRA based on 1) initial experiments showing that WC44 did *not* have a D3 agonist-like profile *in vivo* (Fig. 3), 2) the structural similarities between WC44 and the D3 antagonist/weak partial agonist WC10 (Fig. 1), and 3) similar *in vivo* effects of WC10 and WC44 in a study by Kumar et al. (2009).

2. Methods

2.1. Experimental animals

Adult male SD rats ($n = 184$; 225–250 g; Harlan Laboratories, Livermore, CA) were housed in groups of 2–3 animals per cage, and maintained on a reversed light/dark schedule with water and food available *ad libitum*. Rats were handled within 2 d of arrival. Testing occurred during the dark phase. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by the UCSD Animal Subjects Committee (protocol #S01221). All behavioral testing was completed in a laboratory that is free of all proprietary interests in WC10 or WC44 (MW, WLC, PEP, NRS).

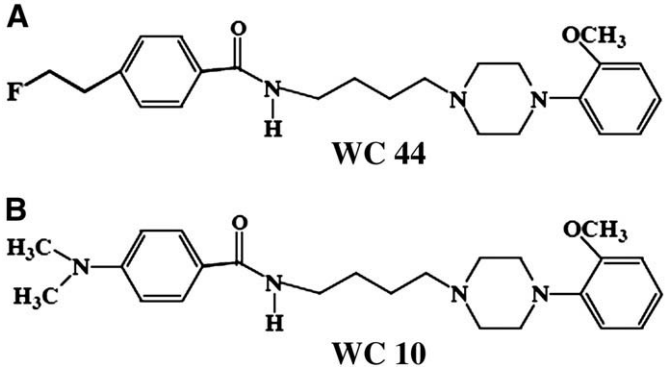

Fig. 1. Structures of the two phenylpiperazine derivates WC44 (A), and WC10 (B).

2.2. Drugs

Apomorphine HCl hemihydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA), PRA from Toronto Research Chemicals (North York, On, Canada), and L741,626 from Tocris (Ellisville, MO, USA). WC10 and WC44 were synthesized by J.P. Durbin according to published methods (structures 12b and 12i, respectively, in Chu et al., 2005). Drug doses are based on mg/kg of salts. APO, PRA, and L741,626 were administered subcutaneously (sc). WC10 and WC44 were administered intra-peritoneally (ip). PRA (0, 1.0 mg/kg) was dissolved in saline, and APO (0, 0.5 mg/kg) was dissolved in 0.01% ascorbate/saline. L741,626 (water vehicle, or 1 mg/kg) was dissolved in 0.05% lactic acid/water (w/v) and pH was adjusted to ≥5 using NaOH. WC10 (5% DMSO/water (v/v) vehicle, 1, 3, or 10 mg/kg) was dissolved in DMSO; water and a few drops of 1 N HCl were added to achieve a final 5%DMSO/water (v/v) (+HCl) solution. WC44 (5% DMSO/water (v/v) vehicle, 1, 3, or 10 mg/kg) was dissolved in DMSO followed by the addition of water to achieve a final 5%DMSO/water (v/v) solution. All injection volumes were 1 ml/kg.

2.3. Apparatus

Startle chambers for rats (San Diego Instruments, San Diego, CA, USA) were housed in a sound-attenuated room, and consisted of a Plexiglas cylinder 8.2 cm in diameter resting on a 12.5×25.5 cm Plexiglas frame within a ventilated enclosure. Noise bursts were presented via a speaker mounted 24 cm above the cylinder. A piezoelectric accelerometer mounted below the Plexiglas frame detected and transduced motion from within the cylinder. Stimulus delivery was controlled by the SR-LAB microcomputer and interface assembly, which also digitized (0-4095), rectified, and recorded stabilimeter readings. One hundred 1-ms readings were collected beginning at stimulus onset. Startle amplitude was defined as the average of the 100 readings.

2.4. Startle testing procedure

Approximately 7 d after shipment arrival, rats were exposed to a short "matching" startle session. They were placed in the startle chambers for a 5 min acclimation period with a 70 dB(A) background noise, and then exposed to a total of 17 P-ALONE trials (40 ms — 120 dB (A) noise bursts) that were interspersed with 3 PREPULSE + PULSE trials in which P-ALONE was preceded 100 ms (onset-to-onset) by a 20 ms noise burst, 12 dB above background. Rats were assigned to drug dose groups based on average %PPI from the matching session to ensure similar baseline PPI levels between groups. Starting 2–5 d after the matching session, drug testing began. One study assessed the effects of WC44 alone, in a one-day study with the WC44 dose (0, 1, 3, 10 mg/kg; i.p.) as the between-subjects factor. Tests of L741,626 (0, 1 mg/kg), WC10 (0, 1, 3, 10 mg/kg), or WC44 (0, 1, 3, 10 mg/kg) vs. APO (0 or 0.5 mg/kg) or PRA (0 or 1 mg/kg) were two day-studies and had a mixed-model, balanced dose-order (vehicle vs. active dose of the agonist) design with L741,626, WC10, or WC44 pretreatments as the between factor and APO or PRA treatments as the within factor.

In the study of WC44 alone, rats were treated with WC44 (0, 1, 3, 10 mg/kg) and placed into the startle chambers 10 min thereafter. Experiments testing L741,626 vs. APO or PRA used a pretreatment time for L741,626 relative to APO or PRA of 30 min. Experiments testing

WC10, WC44 vs. APO or PRA used a pretreatment time of 10 min relative to APO treatment, or 5 min relative to PRA treatment. Rats were placed into the startle chambers immediately after APO treatment or 15 min after PRA treatment. Pretreatment intervals were based on pharmacological data from Kumar et al. (2009), and pilot experiments from our laboratory.

The PPI test session began by placing the rats in the startle chambers followed by a 5 min acclimation period with a 70 dB(A) background noise. Rats were then exposed to a series of trial types, which were presented in pseudorandom order. Interspersed between these active trial types were trials in which no stimulus was presented, but cage displacement was measured (NOSTIM trials). The session consisted of the following trial types: (1) P-ALONE; (2-4) P-ALONE preceded 100 ms (onset-to-onset) by a PREPULSE (PP) consisting of a 20 ms noise burst of either 5, 10, or 15 dB above background (PP5 + PULSE, PP10 + PULSE, or PP15 + PULSE trials, respectively). The session began with 4 consecutive P-ALONE trials and ended with 3 consecutive P-ALONE trials; between these trials were three blocks, each consisting of 8 P-ALONE trials, and 5 trials of each prepulse + pulse combination. Trial blocks were used to assess the time course of drug effects. NOSTIM trials were not included in the calculation of inter-trial intervals. Intertrial intervals were variable and averaged 20 s. Total session duration was 30.5 min.

2.5. Data analysis

PPI was defined as 100-[(startle amplitude on prepulse trials/startle amplitude on P-ALONE trials) × 100], and was analyzed by mixed design ANOVAs. All data was inspected for the presence of "non-responders" defined by a mean startle response to P-ALONE trials of <10 units. Other ANOVAs were used to assess P-ALONE magnitude, or NOSTIM trials. In all cases, analyses of NOSTIM trials revealed expected effects of DA agonists (Mansbach et al., 1988; Weber and Swerdlow, 2008), and no informative interactions with pretreatments (WC10 or WC44), and thus are not reported here in detail. Results from the two test compounds in the APO and PRA assays were directly compared within the same ANOVA in order to base the selection of the most promising compound for subsequent testing and optimization in medical chemistry on a clear scientific rationale. Post-hoc comparisons were conducted using Fisher's PLSD. Data were collapsed across prepulse intensities and PPI blocks. Alpha was 0.05.

3. Results

3.1. L741,626 vs. APO and PRA

In Weber et al. (2008), a threshold dose of 1 mg/kg of the D2 receptor antagonist L741,626 significantly opposed PPI deficits induced by APO, but not those induced by PRA. This finding was confirmed in the present study ($n = 8$ rats/dose of L741,626).

ANOVA of %PPI revealed significant main effects of APO dose ($F = 39.7$, df 1,14, $p < 0.0001$) and L741,626 dose ($F = 15.0$, df 1,14, $p = 0.0017$), and the critical APO x L741,626 dose interaction effect ($F = 5.3$, df 1,14, $p = 0.037$). Post-hoc tests revealed that APO significantly reduced %PPI in rats treated with 0 mg/kg of L741,626 ($p = 0.0013$), and this effect was significantly opposed by 1 mg/kg L741,626 ($p = 0.0045$; Fig. 2A). Yet, in rats treated with 1 mg/kg of L741,626, significant differences between vehicle and APO treated rats remained ($p = 0.0089$), indicating that this dose of L741,626 did not *fully* prevent APO-induced PPI deficits. ANOVA of startle magnitude did not reveal any significant main or interaction effects ($F < 1$ in all cases: inset, Fig. 2A), showing that drugs effects on startle magnitude cannot account for their significant effects on PPI.

In contrast, ANOVA of %PPI revealed a significant main effect of PRA dose ($F = 36.0$, df 1,14, $p < 0.0001$), but not of L741,626 dose ($F < 1$), and no PRA x L741,626 dose interaction ($F = 1.6$, df 1,14, ns; Fig. 2B). ANOVA of startle magnitude revealed a significant main effect of PRA dose ($F = 26.7$, df 1,14, $p < 0.0001$). No main effect of L741,626 dose ($F < 1$), and no PRA x L741,626 interaction ($F = 1.5$, df 1,14, ns; inset, Fig. 2B) were detected. While the magnitude of the PRA-induced suppression of startle magnitude precluded the generation of subgroups matched for startle magnitude, simple regression analysis showed that PRA effects on startle magnitude contributed to less than 1% of the variance of PRA effects on PPI, suggesting that PRA effects on these two measures were independent (regression weights not significant). ANOVAs of %PPI were repeated, adding a factor for low vs. high levels of PRA-induced startle suppression, based on median split analyses. No critical main or interaction effects of %PPI differed between

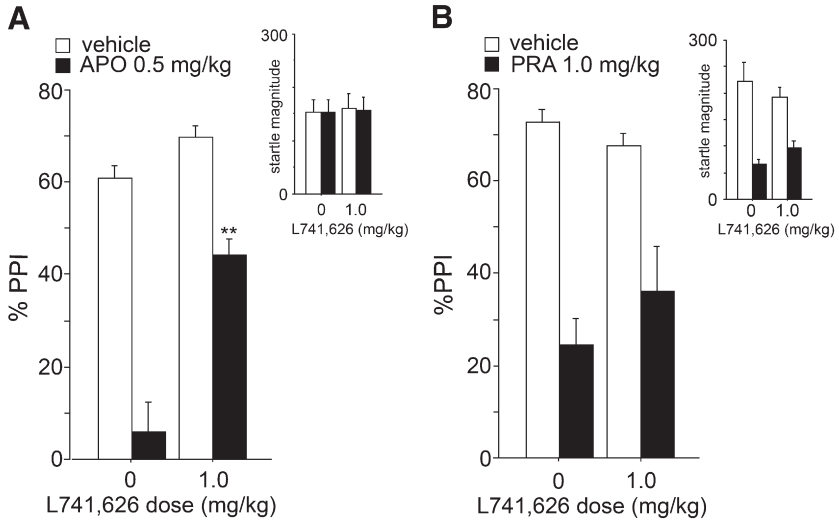

Fig. 2. Effects of a threshold dose of the preferential D2 antagonist L741,626 on PPI deficits induced by APO (A) and PRA (B). (A) ANOVA of %PPI revealed effects of APO ($p < 0.0001$) and L741,626 ($p < 0.005$), and the critical APO x L741,626 interaction ($p < 0.05$). Post-hoc tests revealed that APO reduced %PPI in rats treated with 0 mg/kg of L741,626 ($p < 0.005$), and this effect was opposed by 1 mg/kg L741,626 ($p < 0.005$). ANOVA of startle magnitude did not reveal any main or interaction effects (inset). (B) ANOVA of %PPI revealed an effect of PRA ($p < 0.0001$), but not of L741,626 dose (ns), and no PRA x L741,626 dose interaction effect (ns). ANOVA of startle magnitude revealed a significant main effect of PRA dose ($p < 0.0001$). Drug effects on startle were dissociable from drug effects on PPI (see results). The star symbols denote a significant difference ($p < 0.005$) between APO treated rats pretreated with 0 vs. 1 mg/kg of L741,626.

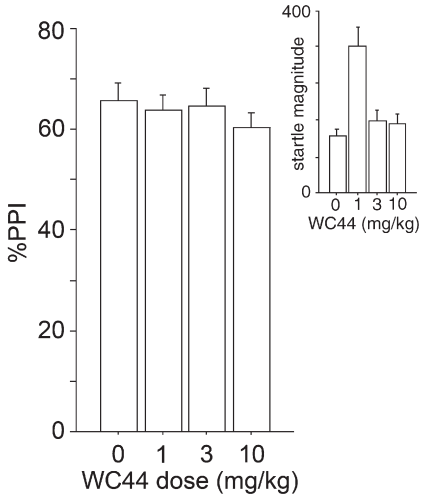

Fig. 3. Effects of the presumed D3 agonist WC44 on PPI and startle magnitude (insets). ANOVA of %PPI in SD rats revealed no significant main effect WC44 dose indicating that WC44 does not have an agonist-like effect *in vivo*. ANOVA of startle magnitude revealed a significant main effect of WC44 dose.

subgroups with low vs. high levels of PRA-induced startle suppression were detected.

3.2. WC44 and WC10 vs. APO and PRA

In vitro assays have characterized WC44 as a full D3 receptor agonist (Chu et al., 2005). We therefore tested the effects of WC44 alone on %PPI ($n=4$/WC44 dose). ANOVA of %PPI revealed no significant main effect WC44 dose ($F<1$; Fig. 3) indicating that WC44 does not have a DA agonist-like effect in this *in vivo* assay. ANOVA of startle magnitude revealed a significant main effect of WC44 dose ($F=4.5$, *df* 3,12, $p=0.025$; Fig. 3 inset), with higher startle magnitudes for 1 mg/kg of WC44 when compared to vehicle ($p=0.030$), but not for any of the other active WC44 doses. Thus, at doses that are bioactive, WC44 does not exhibit DA agonist-like effects on PPI.

We then directly compared the activity of WC10 ($n=8$/WC10 dose) and WC44 ($n=6$/WC44 dose) in the APO assay. ANOVA revealed a significant main effect of APO dose (0 vs. 0.5 mg/kg, $F=183.9$, *df* 1,24, $p<0.0001$), but no main effect of pretreatment type (WC44 vs. WC10; $F=2.3$, *df* 1,24, ns), and no effect of pretreatment dose (0 vs. 10 mg/kg of either test compound; $F=1.6$, *df* 1,24, ns). There were no significant interactions of pretreatment type x pretreatment dose ($F=3.3$, *df* 1,24, $p=0.081$), or pretreatment type x APO ($F<1$, ns). There was a significant interaction of APO x pretreatment dose ($F=4.6$, *df* 1,24, $p=0.042$), and most importantly APO x pretreatment type x pretreatment dose ($F=6.9$, *df* 1,24, $p=0.015$), indicating that WC10 and WC44 differed in their impact on APO-induced PPI deficits. To understand the basis for this interaction, separate ANOVAs were conducted for WC44 and WC10. ANOVA of % PPI for WC44 revealed a significant main effect of APO dose ($F=72.4$, *df* 1,14, $p<0.0001$), but no effect of WC44 dose and no APO x WC44 dose ($F<1$ for all cases) indicating that WC44 does *not* reverse APO-induced PPI deficits. Post-hoc tests revealed that APO significantly reduced %PPI in rats pretreated with 0 mg/kg of WC44 ($p=0.0011$) and in rats pretreated with 10 mg/kg WC44 ($p=0.0026$), and that among APO-treated rats, %PPI did not differ between those pretreated with 0 vs. 10 mg/kg WC44 (ns). ANOVA of %PPI for WC10 revealed a significant main effect of APO dose ($F=115.2$, *df* 1,14, $p<0.0001$) and WC10 dose ($F=5.8$, *df* 1,14, $p=0.031$), and most importantly a significant APO x WC10 interaction ($F=16.5$, *df* 1,14, $p=0.0012$). Post-hoc tests revealed that APO significantly reduced %PPI in rats treated with 0 mg/kg of WC10 ($p<0.0001$), and this effect was significantly opposed by 10 mg/kg WC10 ($p=0.0051$), indicating that WC10 opposed APO-induced PPI deficits (Fig. 4A). Yet, in rats treated with 10 mg/kg of WC10 a significant difference between rats treated with vehicle and APO remained ($p=0.0042$), indicating that this dose of WC10 did not *fully* prevent PRA-induced PPI deficits.

ANOVA of startle magnitude revealed a significant main effect of pretreatment type (WC44 vs. WC10, $F=8.7$, *df* 1,24, $p=0.007$), reflecting reduced startle amplitudes in the WC10 experiment. All other effects were not statistically significant (Fig. 4A inset). Importantly, the effect of pretreatment type on startle magnitude is neither due to APO, WC10, WC44, nor a combination thereof and therefore cannot account for the critical interaction of APO x pretreatment type x pretreatment dose in PPI

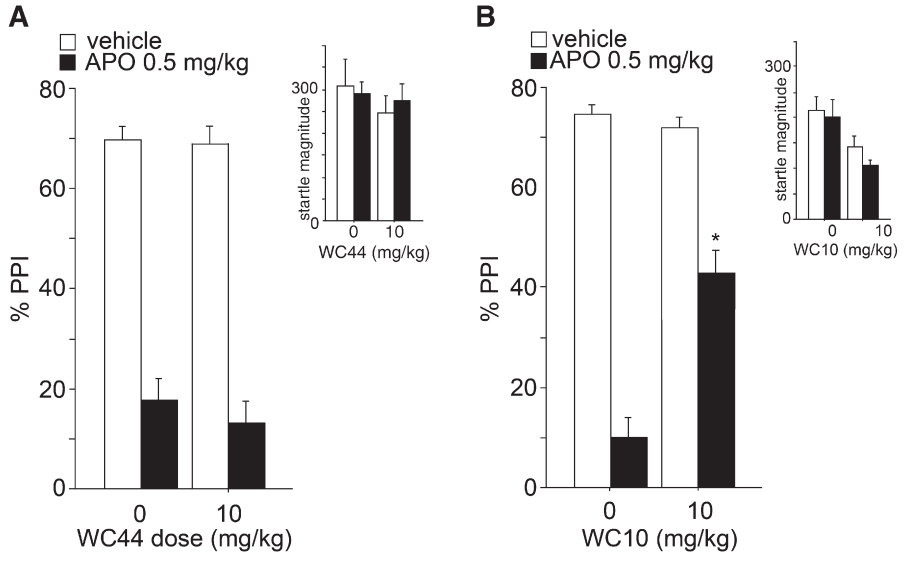

Fig. 4. Effects of WC44 (A) and WC10 (B) on PPI deficits induced by APO. ANOVA of %PPI revealed an APO dose effect ($p<0.0001$), an APO x pretreatment-dose effect ($p<0.05$), and an APO dose x pretreatment dose x pretreatment type effect ($p<0.05$). Separate ANOVAs for the WC44 and WC10 experiment revealed a main effect of APO in both experiments ($p<0.0001$, each). In addition, an APO dose x pretreatment dose effect for WC10 ($p<0.005$), but not for WC44 (n.s.), indicative of reversal of APO-induced PPI deficits in rats treated with WC10, but not WC44. ANOVA of startle magnitude revealed a main effect of pretreatment type ($p<0.01$) indicating lower startle magnitudes in the WC10 experiment; all drug-related main or interaction effect were non-significant, indicating that drug effects on PPI were dissociable from drug effects on startle magnitude. The star symbol denotes significant differences ($p<0.05$) between APO rats pre-treated with 0 vs. 10 mg/kg of WC10.

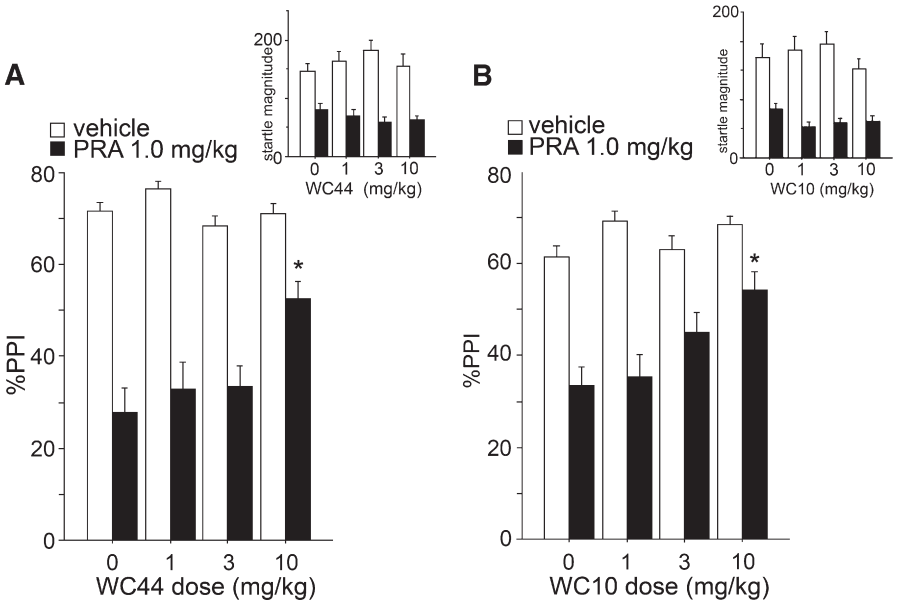

Fig. 5. Effects of WC44 (A) and WC10 (B) on PPI deficits induced by PRA, and startle magnitude (insets). ANOVA of %PPI revealed effects of pretreatment dose ($p<0.05$), PRA dose ($p<0.0001$), PRA x pretreatment type effect ($p<0.05$) and the crucial PRA x pretreatment dose effect ($p<0.05$), but no PRA x pretreatment type x pretreatment dose effect (n.s.). ANOVA of startle magnitude revealed a PRA dose effect, but no main effect or interaction effect with pretreatment dose, or pretreatment type, indicating that drug effects on startle magnitude cannot account for the prevention of PRA-induced PPI deficits by either test compound. Star symbols denote significant differences ($p<0.05$) between PRA treated rats pre-treated with 0 vs. 10 mg/kg of either WC10 or WC44.

measures described above. Concurrently, in subsets of animals from both experiments that were matched for mean startle magnitude (effect of experiment: $F<1$), the critical interaction of APO x pretreatment type x pretreatment dose in measures of PPI was still apparent ($p=0.014$), with identical post-hoc patterns to those detected in the inclusive sample.

Parallel analyses were completed ($n=12$/WC10 dose and $n=12$/WC44 dose) using PRA to disrupt PPI instead of APO. ANOVA revealed a significant main effect of PRA dose (0 vs. 1 mg/kg, $F=46.9$, *df* 1,44, $p<0.0001$), and pretreatment dose (0 vs. 10 mg/kg of either test compound; $F=9.5$, *df* 1,44, $p=0.0035$), but no main effect of pretreatment type (WC44 vs. WC10; $F<1$). Importantly, there was a significant effect of PRA x pretreatment dose ($F=6.5$, *df* 1,44, $p=0.015$), but no interaction of PRA x pretreatment type ($F=1.7$, *df* 1,44, ns), no pretreatment type x pretreatment dose interaction ($F<1$), and no PRA x pretreatment type x pretreatment dose interaction ($F<1$), indicating that the two test compounds did not differ significantly from each other in their ability to reverse these PRA-induced PPI deficits. A post-hoc comparison in PRA-treated rats across compounds revealed that, compared to the 0 mg/kg pretreatment dose, the 10 mg/kg dose significantly increased PPI ($F=9.9$, *df* 1,46, $p=0.0029$). Separate ANOVAs were then conducted for WC44 and WC10. ANOVA of %PPI for WC44 revealed a significant main effect of PRA dose ($F=26.6$, *df* 1,22, $p<0.0001$), a trend towards a WC44 dose effect ($F=3.5$, *df* 1,22, $p=0.073$), and a significant PRA x WC44 dose interaction ($F=4.5$, *df* 1,22, $p=0.047$), reflecting the fact that WC44 does reverse PRA-induced PPI deficits. Post-hoc tests revealed that PRA significantly reduced %PPI in rats treated with 0 mg/kg of WC44 ($p=0.0011$), and this effect was opposed by 10 mg/kg WC44 ($p=0.045$). Yet, in rats treated with 10 mg/kg of WC44, significant differences between vehicle and PRA treated rats remained ($p=0.018$), indicating that this dose of WC44 did not *fully* prevent PRA-induced PPI deficits. ANOVA of %PPI for WC10 revealed a significant main effect of PRA dose ($F=20.4$, *df* 1,22, $p=0.0002$) and WC10 dose ($F=6.4$, *df* 1,22, $p=0.019$), while the PRA x WC10 interaction effect did not reach significance ($F=2.0$, *df* 1,22, ns). Post-hoc tests, however, revealed that PRA significantly reduced %PPI in rats treated with 0 mg/kg of WC10 ($p=0.0004$), and this effect was significantly opposed by 10 mg/kg WC10 ($p=0.032$; Fig. 5A). Similarly to the results obtained with WC44, however, in rats treated with 10 mg/kg of WC10, there was a trend towards differences between vehicle and PRA treated rats ($p=0.082$), suggesting that this dose of WC10 did not *fully* prevent PRA-induced PPI deficits.

The corresponding ANOVA of startle magnitude based on the comparison of 0 vs. 10 mg/kg of the test compounds revealed a significant main effect of PRA dose ($F=56.1$, *df* 1,44, $p<0.0001$). All other main or interaction effects were not statistically significant (Fig. 5B inset). While the magnitude of the startle suppression induced by PRA did not allow to create subgroups of rats matched for startle magnitude, simple regression analyses based on the comparison of 0 vs. 10 mg/kg of the test compounds showed that the PRA effect on startle amplitude accounted for less than 2% of the corresponding PPI effects, suggesting that these two measures were independent (regression weights ns). To further assess this issue, all ANOVAs of %PPI were repeated, adding a factor for low vs. high levels of PRA-induced startle suppression, based on median split analyses. No critical main or interaction effects of %PPI differed between subgroups with low vs. high levels of PRA-induced startle suppression.

4. Discussion

In the present study, measures of PPI deficits were used to identify novel test compounds that act as preferential D3-receptor linked antagonists in rats. WC10 and WC44, two representatives of a novel panel of putative D3 selective compounds, were characterized in this assay. A D1/D2/(D3) related mechanism of action was identified for WC10. A novel preclinical profile based on a functional D3-receptor antagonist mechanism of action was identified for WC44.

Many groups have demonstrated that APO-induced PPI deficits are opposed by typical and atypical antipsychotics (c.f. Swerdlow et al., 2008). We have also reported PPI-disruptive effects of the preferential D3 receptor agonist PRA in rats (Weber et al., 2008). This effect was confirmed in the present study, consistent with studies using other preferential D3 agonists (Caine et al., 1995; Swerdlow et al., 1998; Varty and Higgins, 1998; Zhang et al., 2007). Our previous studies have further demonstrated a greater sensitivity of the preferential D2

receptor antagonist L741,626 to reverse APO-induced PPI deficits vs. PRA-induced PPI deficits, suggesting that, unlike APO, PRA effects on PPI are not mediated by D2 receptors (Weber et al., 2008). These findings were also confirmed here (Fig. 2). The present study used these apparent differences in D3 vs. D2 involvement in PRA- vs. APO-induced PPI deficits to characterize WC10 and WC44, two novel D3-receptor selective compounds, and to predict novel preclinical antipsychotic profiles consistent with a predominant D3 receptor-linked mechanism of action. Zhang et al. (2007) used a similar strategy to compare the effects of preferential D3 antagonists in assays of PPI deficits induced by either APO, or the preferential D3 agonist PD128,907 in rats. As large clinical trials for D3 preferential antagonists have not been published to date, we cannot conclude that the high antipsychotic predictive validity of the APO/PPI for mixed D2/D3 antagonists extends to the PRA/PPI assay for the detection of potentially novel antipsychotics with a preferential D3-receptor linked mechanism of action. However, at the very least, our findings and those of Zhang et al. (2007) show that this use of the PPI assays can detect apparent differences in functional D3 antagonism *in vivo*.

WC10 and WC44 had very distinct profiles in the APO-PPI assay: WC10 significantly opposed APO-induced PPI deficits, while WC44 did not. This profile of WC10 is shared with both typical and atypical antipsychotics (Swerdlow et al., 1994; c.f. Geyer et al., 2001; Swerdlow et al., 2008), while the inactivity of WC44 in this assay parallels that reported with the preferential D3 antagonists A-691990 and SB-277011 (Zhang et al., 2007). The prevention of APO-induced PPI deficits *per se* does not rule out a D3-receptor related mechanism of action, as the PPI-disruption caused by a APO is likely to involve both D3 and D2, as well as other DA receptor subtypes (for the receptor binding profile of APO see e.g.: Millan et al., 2002). Consistent with this, Millan et al. (2008) reported that the highly preferential D3 antagonist S33138 significantly opposed APO-induced PPI deficits in rats. To our knowledge, no study has yet compared the effects of S33138 on PPI deficits induced by APO- vs. those induced by a preferential D3 agonist, like PRA, PD128,907, or 7-OHDPAT.

In the PRA/PPI assay, both WC10 and WC44 opposed PRA-induced PPI deficits. Previous studies have shown that PPI deficits induced by preferential D3 agonists such as PD128,907 and 7-OHDPAT can be prevented by preferential D3 receptor antagonists such as A-691990 and SB-277011 (Zhang et al., 2007) as well as by the mixed D2-like antagonist such as HAL (Caine et al., 1995; Zhang et al., 2007). This shows that the prevention of PRA-induced PPI-deficits per se does not indicate a novel antipsychotic-like profile. *However, the combination of inactivity in the APO/PPI assay, and activity in the PRA/PPI assay, suggest that WC44 has properties of a novel, D3 preferential antagonist*. Arguably, no full reversal of the PRA-induced PPI deficits was achieved with either test compound in the dose range tested, suggesting that higher doses should be tested (see below), or that more efficacious compounds are needed. We are currently studying variants of WC44 with the goal to achieve increased efficacy in this model.

In the *in vitro* assay used by Chu et al. (2005), the effects of WC44 were tested in the absence of DA, using forskolin-stimulated adenylate cyclase activity; WC44 yielded 92% of the response induced by quinpirole and was hence classified as a full D3 agonist. The contrast of the agonist-like profile for WC44 in these *in vitro* studies vs. the lack of agonist-like effects in the *in vivo* findings reported here (Fig. 3) is intriguing. We cannot exclude the possibility that WC44 could have DA agonist-like effects at a higher dose range, but the range used here includes the IC_{50} of 5.5 mg/kg for the suppression of abnormal involuntary movements (AIM) in the same rat strain (Kumar et al., 2009). In ongoing studies, we have detected no independent effects of 20 mg/kg WC44 on PPI, but a near-complete opposition of PRA-induced PPI deficits (in preparation). While we have no immediate explanation for the lack of agonist-like *in vivo* effects of WC44 at present, one explanation may be the presence of endogenous DA *in vivo*. Among all DA receptors, D3 receptors have the highest DA affinity with a K_i in the range of extrasynaptic and intrasynaptic DA levels (cf. Richtand, 2006). This suggests that D3 receptors are likely to be (partially) occupied by DA *in vivo*. Hence, agonist-like effects derived under *in vitro* conditions in the absence of DA may not translate to *in vivo* findings for compounds with (even weak) partial agonist-like properties relative to DA. A second explanation may lie in the concept of functional selectivity, i.e. the phenomenon that a compound acting via a single receptor type can have diverse functional properties ranging from full agonist, to partial agonist, to full antagonist, depending on the intracellular signaling cascade that is activated downstream of the receptor (Mailman, 2007).

While both WC10 and WC44 have been thoroughly characterized in *in vitro* binding studies for DA, serotonin, and sigma receptors, and in functional assays for D2/D3 receptors (Chu et al., 2005), it is not known at present whether these compounds have functional activity on non-DA receptors. For example, a relatively high binding affinity was detected for 5-HT1a receptors for both compounds, but the functional consequences of these binding properties are not yet known. 5-HT1a agonists disrupt PPI in rats (Rigdon and Weatherspoon, 1992), but neither WC10, nor WC44 disrupted PPI in the present study. Thus, the most parsimonious explanation of the present data is that the effects detected with WC10 and WC44 reflect DA-linked mechanisms of these test compounds.

In summary, studies detected D2 and D3 antagonist profiles for WC10, and findings suggested that WC44 may functionally oppose D3 but not D2 receptor activation. Based on this latter profile, WC44 might have a novel antipsychotic profile linked to functional D3 receptor antagonism. These initial studies cannot rule out a potential role of other receptors types for which the binding affinities of WC10 and WC44 are not yet known. Other test compounds with putative functional D3 antagonism are being evaluated in this assay, with the goal of predicting their potential for clinical applications.

Acknowledgements

The authors gratefully acknowledge M. Bongiovanni for assistance in manuscript preparation, and M. Breier, D. Ko, N. Thangaraj and D. Marzan for technical assistance. Research was supported by the National Alliance for Research on Schizophrenia and Depression, the Tourette Syndrome Association, and VISN22 MIRECC (MW), DA23957 (RL). MH59803 (NRS) and MH68366 (NRS). In the past 3 years, NRS received support from Allergan Pharmaceuticals (research funding, consultancy funding) and Sanofi/Aventis (consultancy).

References

Braff D, Stone C, Callaway E, Geyer M, Glick I, Bali L. Prestimulus effects on human startle reflex in normals and schizophrenics. Psychophysiology 1978;15:339–43.

Cadenhead KS, Swerdlow NR, Shafer KM, Diaz M, Braff DL. Modulation of the startle response and startle laterality in relatives of schizophrenic patients and in subjects with schizotypal personality disorder: evidence of inhibitory deficits. Am J Psychiatry 2000;157:1660–8.

Caine SB, Geyer MA, Swerdlow NR. Effects of D3/D2 dopamine receptor agonists and antagonists on prepulse inhibition of acoustic startle in the rat. Neuropsychopharmacology 1995;12:139–45.

Chu W, Tu Z, McElveen E, Xu J, Taylor M, Luedtke RR, et al. Synthesis and in vitro binding of N-phenyl piperazine analogs as potential dopamine D3 receptor ligands. Bioorg Med Chem 2005;13:77–87.

Geyer MA, Krebs-Thomson K, Braff DL, Swerdlow NR. Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. Psychopharmacology 2001;156:117–54.

Giakoumaki SG, Roussos P, Frangou S, Bitsios P. Disruption of prepulse inhibition of the startle reflex by the preferential D(3) agonist ropinirole in healthy males. Psychopharmacology 2007;194:289–95.

Gurevich EV, Bordelon Y, Shapiro RM, Arnold SE, Gur RE, Joyce JN. Mesolimbic dopamine D3 receptors and use of antipsychotics in patients with schizophrenia. A postmortem study. Arch Gen Psychiatry 1997;54:225–32.

Joyce JN, Millan MJ. Dopamine D3 receptor antagonists as therapeutic agents. Drug Discov Today 2005;10:917–25.

Kumar R, Riddle LR, Griffin SA, Chu W, Vangveravong S, Neisewander J, et al. Evaluation of D2 and D3 dopamine receptor selective compounds on L-dopa-dependent abnormal involuntary movements in rats. Neuropharmacology 2009;56:956–69.

Kumari V, Das M, Zachariah E, Ettinger U, Sharma T. Reduced prepulse inhibition in unaffected siblings of schizophrenia patients. Psychophysiology 2005;42:588–94.
Luedtke RR, Mach RH. Progress in developing D3 dopamine receptor ligands as potential therapeutic agents for neurological and neuropsychiatric disorders. Curr Pharm Des 2003;9:643–71.
Mailman RB. GPCR functional selectivity has therapeutic impact. Trends Pharmacol Sci 2007;28:390–6.
Mansbach RS, Geyer MA, Braff DL. Dopaminergic stimulation disrupts sensorimotor gating in the rat. Psychopharmacology 1988;94:507–14.
Millan MJ, Loiseau F, Dekeyne A, Gobert A, Flik G, Cremers TI, et al. S33138 (N-[4-[2-[(3aS,9bR)-8-cyano-1,3a,4,9b-tetrahydro[1] benzopyrano[3,4-c]pyrrol-2(3H)-yl)-ethyl]phenyl-acetamide), a preferential dopamine D3 versus D2 receptor antagonist and potential antipsychotic agent: III. Actions in models of therapeutic activity and induction of side effects. J Pharmacol Exp Ther 2008;324:12-1226.
Millan MJ, Maiofiss L, Cussac D, Audinot V, Boutin JA, Newman-Tancredi A. Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. I. A multivariate analysis of the binding profiles of 14 drugs at 21 native and cloned human receptor subtypes. J Pharmacol Exp Ther 2002;303:791–804.
Richtand NM. Behavioral sensitization, alternative splicing, and d3 dopamine receptor-mediated inhibitory function. Neuropsychopharmacology 2006;31:2368–75.
Rigdon GC, Weatherspoon JK. 5-Hydroxytryptamine 1a receptor agonists block prepulse inhibition of acoustic startle reflex. J Pharmacol Exp Ther 1992;263:486–93.
Roussos P, Giakoumaki SG, Bitsios P. The dopamine D(3) receptor Ser9Gly polymorphism modulates prepulse inhibition of the acoustic startle reflex. Biol Psychiatry 2008;64:235–40.
Sokoloff P, Diaz J, Le Foll B, Guillin O, Leriche L, Bezard E, et al. The dopamine D3 receptor: a therapeutic target for the treatment of neuropsychiatric disorders. CNS Neurol Disord Drug Targets 2006;5:25–43.
Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC. Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 1990;347:146–51.
Swerdlow NR, Braff DL, Taaid N, Geyer MA. Assessing the validity of an animal model of deficient sensorimotor gating in schizophrenic patients. Arch Gen Psychiatry 1994;51:139–54.
Swerdlow NR, Braff DL, Geyer MA, Koob GF. Central dopamine hyperactivity in rats mimics abnormal acoustic startle response in schizophrenics. Biol Psychiatry 1986;21:23–33.
Swerdlow NR, Light GA, Cadenhead KS, Sprock J, Hsieh MH, Braff DL. Startle gating deficits in a large cohort of patients with schizophrenia: relationship to medications, symptoms, neurocognition, and level of function. Arch Gen Psychiatry 2006;63:1325–35.
Swerdlow NR, Taaid N, Oostwegel JL, Randolph E, Geyer MA. Towards a cross-species pharmacology of sensorimotor gating: effects of amantadine, bromocriptine, pergolide and ropinirole on prepulse inhibition of acoustic startle in rats. Behav Pharmacol 1998;9:389–96.
Swerdlow NR, Weber M, Qu Y, Light GA, Braff DL. Realistic expectations of prepulse inhibition in translational models for schizophrenia research. Psychopharmacology 2008;199:331–88.
Varty GB, Higgins GA. Dopamine agonist-induced hypothermia and disruption of prepulse inhibition: evidence for a role of D3 receptors? Behav Pharmacol 1998;9:445–55.
Weber M, Chang WL, Breier M, Ko D, Swerdlow NR. Heritable strain differences in sensitivity to the startle gating-disruptive effects of D2 but not D3 receptor stimulation. Behav Pharmacol 2008;19:786–95.
Weber M, Swerdlow NR. Rat strain differences in startle gating-disruptive effects of apomorphine occur with both acoustic and visual prepulses. Pharmacol Biochem Behav 2008;88:306–11.
Zhang M, Ballard ME, Unger LV, Haupt A, Gross G, Decker MW, et al. Effects of antipsychotics and selective D3 antagonists on PPI deficits induced by PD 128907 and apomorphine. Behav Brain Res 2007;182:1-11.