Asenapine Increases Dopamine, Norepinephrine, and Acetylcholine Efflux in the Rat Medial Prefrontal Cortex and Hippocampus

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Atypical antipsychotic drugs, which are more potent direct acting antagonists of brain serotonin (5-HT)_{2A} than dopamine (DA) D₂ receptors, preferentially enhance DA and acetylcholine (ACh) efflux in the rat medial prefrontal cortex (mPFC) and hippocampus (HIP), compared with the nucleus accumbens (NAc). These effects may contribute to their ability, albeit limited, to improve cognitive function and negative symptoms in patients with schizophrenia. Asenapine (ASE), a new multireceptor antagonist currently in development for the treatment of schizophrenia and bipolar disorder, has complex serotonergic properties based upon relatively high affinity for multiple serotonin (5-HT) receptors, particularly 5-HT_{2A} and 5-HT_{2C} receptors. In the current study, the effects of ASE on DA, norepinephrine (NE), 5-HT, ACh, glutamate, and γ -aminobutyric acid (GABA) efflux in rat mPFC, HIP, and NAc were investigated with microdialysis in awake, freely moving rats. ASE at 0.05, 0.1, and 0.5 mg/kg (s.c.), but not 0.01 mg/kg, significantly increased DA efflux in the mPFC and HIP. Only the 0.5 mg/kg dose enhanced DA efflux in the NAc. ASE, at 0.1 and 0.5 mg/kg, significantly increased ACh efflux in the mPFC, but only the 0.5 mg/kg dose of ASE increased HIP ACh efflux. ASE did not increase ACh efflux in the NAc at any of the doses tested. The effect of ASE (0.1 mg/kg) on DA and ACh efflux was blocked by pretreatment with WAY100635, a 5-HT_{1A} antagonist/D₄ agonist, suggesting involvement of indirect 5-HT_{1A} agonism in both the actions. ASE, at 0.1 mg/kg, increased NE, but not 5-HT, efflux in the mPFC and HIP. ASE, at 0.1 mg/kg (s.c.), had no effect on glutamate and GABA efflux in either the mPFC or NAc. These findings indicate that ASE is similar to clozapine and other atypical antipsychotic drugs in preferentially increasing the efflux of DA, NE, and ACh in the mPFC and HIP compared with the NAc, and suggests that, like these agents, it may also improve cognitive function and negative symptoms in patients with schizophrenia.

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INTRODUCTION

Atypical antipsychotics differ from conventional antipsychotic drugs in that they cause fewer extrapyramidal symptoms (EPS), do not produce sustained elevations of serum prolactin levels (with the exception of risperidone and paliperidone), and can improve some domains of cognition in patients with schizophrenia (Meltzer and McGurk, 1999; Harvey and Keefe, 2001; Woodward *et al*, 2005; Keefe *et al*, 2007). The basis for the cognitive impairment in schizophrenia is complex; it has principally

been related to diminished or dysregulated brain dopaminergic (Weinberger *et al*, 1988), noradrenergic (Arnsten and Li, 2005), cholinergic (Meltzer and McGurk, 1999; Bymaster *et al*, 2002), glutamatergic (Hirsch *et al*, 1997), and γ -aminobutyric acid (GABA) activity (Benes and Berretta, 2000), to neuronal or neuropil loss (Selemon and Goldman-Rakic, 1999), and to abnormalities in connectivity (Pantelis *et al*, 1997; Nakamura *et al*, 2005). Thus, the ability of atypical antipsychotic drugs to preferentially increase extracellular efflux of dopamine (DA), norepinephrine (NE), and acetylcholine (ACh) in the medial prefrontal cortex (mPFC) and hippocampus (HIP) has been postulated to contribute to their ability to improve cognition, in schizophrenia, and possibly negative symptoms and depression (Assie *et al*, 2005; Devoto *et al*, 2004; Ichikawa *et al*, 1998; Ichikawa *et al*, 2002a, b, c; Kuroki *et al*, 1999; Zhang *et al*, 2000). The effects of atypical antipsychotic drugs on glutamatergic transmission has also been suggested to

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contribute to their ability to improve cognitive impairment in schizophrenia (Konradi and Heckers, 2003; Coyle, 2006). Acute treatment with clozapine has been reported to enhance extracellular glutamate levels while decreasing GABA efflux in the rat mPFC, in some but not all studies (Bourdelaïs and Deutch, 1994; Daly and Moghaddam, 1993; Yamamoto *et al*, 1994; Yamamoto and Cooperman, 1994; Heidbreder *et al*, 2001). Olanzapine, however, at 5 mg/kg, had no effect on the extracellular levels of glutamate, whereas clozapine, at 10 mg/kg, significantly increased extracellular glutamate levels in the same study (Heidbreder *et al*, 2001). Haloperidol has also been reported to decrease extracellular GABA efflux from interneurons in the mPFC (Bourdelaïs and Deutch, 1994).

Asenapine (ASE) (Org 5222; *trans*-5-chloro-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7,5]-oxepino-[4,5c]pyrrolidine maleate) is a putative atypical antipsychotic drug (Broekkamp *et al*, 1990; Bymaster *et al*, 1996; Costall *et al*, 1990; De Boer *et al*, 1990, 1993; Shahid *et al*, 2007) currently being developed for the treatment of schizophrenia and bipolar disorder (Alphs *et al*, 2007). Potkin *et al* (2007) have recently reported that ASE improves positive and negative symptoms in patients with schizophrenia at least as effectively as risperidone (Potkin *et al*, 2007).

In vitro and *in vivo* receptor binding studies have shown that ASE has high affinities for multiple monoamine receptors, including (1) D_{1,2,3} and D₄; (2) 5-HT_{2A}, 2C, 1A, 1B, 2B, 5A, 6, 7; (3) adrenergic α_{1A} and $\alpha_{2A,2B,2C}$; and (4) histamine H₁ and H₂ receptors, but that it lacks affinity for muscarinic receptors (Bymaster *et al*, 1996; Cosi and Koek, 2001; Matsubara *et al*, 1993; Prinssen *et al*, 2000; Richelson and Souder, 2000; Schotte *et al*, 1996; Shahid *et al*, 2007). *In vitro* assessments have shown that ASE is an antagonist at all of the monoamine receptors listed above (Shahid *et al*, 2007). ASE has approximately 20-fold higher affinity for 5-HT_{2A} compared with DA D₂ receptors (De Boer *et al*, 1993; Schotte *et al*, 1996; Shahid *et al*, 2007). Shahid *et al* (2007) reported the pK_i for ASE for the 5-HT_{2A} receptor as 10.15, and for the D_{2L}, 8.90, using cloned human receptors stably expressed in mammalian cell lines. The occupancy in the rat brain of cortical 5-HT_{2A} receptors by ASE is also about 20-fold higher than that of striatal D₂ receptors (Schotte *et al*, 1996). The relatively high occupancy of 5-HT_{2A} compared with the D₂ receptor may be critically important for some atypical antipsychotic drug actions, such as low EPS and efficacy in treatment-resistant schizophrenia (Meltzer *et al*, 1989, 2003, 2008). This profile has also been proposed as an important component of the preferential release of cortical DA by relatively specific ligands for these receptors, for example, M 100907, SR 43469B, or ACP-103, and haloperidol (Liegeois *et al*, 2002; Bonaccorso *et al*, 2002; Li *et al*, 2005), as well as clozapine, olanzapine, risperidone, and other atypical antipsychotic drugs, which are more potent 5-HT_{2A} than D₂ antagonists (Kuroki *et al*, 1999).

The ability of WAY100635, a silent 5-HT_{1A} antagonist and D₄ agonist (Chemel *et al*, 2006), to block most or all of the ability of the atypical antipsychotic drugs to enhance cortical DA (Ichikawa *et al*, 2001; Li *et al*, 2004), as well as, for some, for example, quetiapine, ACh efflux (Ichikawa *et al*, 2002a, c; Sato *et al*, 2007), suggests that these effects of the atypical antipsychotic drugs are due in part to indirect or direct 5-HT_{1A} agonism (Ichikawa *et al*, 2001; Meltzer

et al, 2003; Li *et al*, 2004). Risperidone and olanzapine are atypical antipsychotic drugs whose effects on cortical DA efflux are blocked by WAY100635, but which themselves are not 5-HT_{1A} agonists. Aripiprazole, bifeprunox, clozapine, quetiapine, and ziprasidone, on the other hand, are 5-HT_{1A} partial agonists (Cosi and Koek, 2001). We, therefore, sought to characterize the effect of WAY100635 on ASE-induced increases in DA and ACh efflux in mPFC. We also sought to characterize the effect of ASE on the efflux of cortical 5-HT, glutamate, and GABA, as there are limited data on the ability of atypical antipsychotic drugs to affect the efflux of these important neurotransmitters.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley albino rats (Zivic-Miller Laboratories, Porterville, PA) weighing 250–300 g were used throughout the study. They were housed two per cage in a controlled 12:12-h light-dark cycle under constant temperature at 22°C, with free access to food and water.

Surgery and Microdialysis

Rats were anesthetized with a combination of intraperitoneal chloral hydrate (172 mg/kg) and pentobarbital (35.6 mg/kg), and mounted in a stereotaxic frame (Stoetling, Wood Dale, IL). A stainless steel guide cannula (21 G) with a dummy probe was placed and fixed by cranioplastic cement (Plastics One, Roanoke, VA) onto the cortex, dorsal to the mPFC. The stereotaxic coordinates for the implanted probe were A, +3.2 mm; L, –0.8 mm (10° inclination); V, –5.5 mm, relative to the bregma. The coordinates for HIP and nucleus accumbens (NAc) were A, –5.6 mm; L, +5 mm; V, –7 mm; and A, +2 mm; L, +1.5 mm; V, –7.5 mm, respectively. The incisor bar level was –3 mm, according to the atlas of Paxinos and Watson (1986). Concentric-shaped dialysis probes were constructed as described elsewhere (Ichikawa *et al*, 2001). A hollow-fiber dialysis membrane (polyacrylonitrile/sodium methylsulfonate polymer, 310 μ m, o.d., 220 μ m, i.d., molecular weight cut-off 40 000Da; AN69 HF; Hôpital SA, Lyon, France) was used, with 2 mm of non-glued surface exposed for dialyzing. A total of 3–5 days after cannulation, dual dialysis probes were implanted into the rat mPFC and HIP/NAc under light anesthesia with methylsulfonate (Metofane; Pitman-Moore, Mundelein, IL). For systemic administration of drugs or the vehicle, a microbore Tygon tubing (TGY-010, 0.03 inches, o.d., 0.01 inches, i.d.; Small Parts Inc., Miami Lakes, FL) catheter was implanted subcutaneously into the intrascapular space. Rats were then housed individually in dialysis cages, with overnight perfusion (0.3 μ l/min) of the probe, with the perfusion rate increased to 1.5 μ l/min on the morning of the day of dialysis. After 1 h of perfusion at 1.5 μ l/min, dialysate samples were collected every 30 min for measuring dialysate neurotransmitter concentrations. The perfusion medium was Dulbecco's phosphate-buffered saline solution (Sigma, St Louis, MO), including Ca²⁺ (138 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂, 1.2 mM CaCl₂, pH = 7.4). After stable baseline values were obtained in the dialysates, each

drug or vehicle was administered subcutaneously. The effect of the drug on neurotransmitter release was monitored for another 180 min. Locations of dialysis probes were verified at the end of each experiment by manual brain dissection and using 100- μ m brain slices (OTS-4000; FHC Inc., Bowdoinham, ME). The procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of Vanderbilt University, Nashville, TN.

Biochemical Assays

DA, NE, serotonin, and ACh analysis. All samples were directly applied onto a high-performance liquid chromatography (HPLC) system with electrochemical detection. DA and NE were simultaneously separated on a reversed-phase column (Xtera RP18, 3 μ m, 2.1 $\times$ 100 mm; Waters Co., Milford, MA). The mobile phase consisted of buffer (24 mM anhydrous citric acid, 48 mM sodium acetate trihydrate, 2 mM sodium dodecylsulfate, 0.5 mM EDTA-2Na), acetonitrile, and methanol in a ratio of 88:8:4, respectively, and adjusted to pH 4.8 with sodium hydroxide. The flow rate was 0.3 ml/min. The potentials for Coulochem microdialysis electrode (ESA 5014B; ESA Biosciences Inc., Chelmsford, MA) were -100 mV and 180 mV vs an Ag/AgCl reference electrode. The retention times for NE and DA were 4.6 and 13 min, respectively. The method for determination of dialysate 5-HT has been described previously (Ichikawa *et al*, 1998). The method for determination of dialysate ACh, without addition of a cholinesterase inhibitor, has been described previously (Ichikawa *et al*, 2002b).

Glutamate and GABA analysis. The stock-derivatizing agent OPA (*o*-phthalaldehyde, 3.0 mg; Sigma-Aldrich, St Louis, MO) was dissolved in 140 μ l absolute ethanol (HPLC grade), to which was added 140 μ l sodium sulfite (Na_2SO_3 , 1 mM) and 2.4 ml of sodium tetraborate buffer at pH 10.4 (NaBH_4 , 1 mM). The working OPA consisted of 0.5 ml stock OPA, 0.5 ml Na_2SO_3 , and 4 ml NaBH_4 (pH 10.4). β -Aminobutyric acid dissolved in water constituted the internal standard. The reaction of 5 μ l internal standard, 20 μ l sample, and 5 μ l working OPA solution proceeded at room temperature for 30 min before injection into the HPLC system. The column system consisted of a small column (XTerra reverse-phase C18, 2.1 $\times$ 10 mm, column A; Waters Co.) and a main reverse-phase column (Nova-Pak C18, 3.9 $\times$ 300 mm, 5 μ m, column B; Waters Co.) kept at 36°C when in use. The mobile phase consisted of 83.5% (v/v) of buffer (0.1 M sodium dihydrogen phosphate dehydrate, 2 mM tetrabutylammonium phosphate, and 1 mM EDTA, adjusted to pH 4 with 1 M phosphoric acid) and 16.5% of acetonitrile, perfused at a flow rate of 0.9 ml/min by a gradient liquid chromatograph pump (LC-10AD; Shimadzu Corp., Kyoto, Japan). A high-density glassy carbon working electrode combined with an Ag/AgCl reference electrode was operated at $+0.58$ V. A Rheodyne injection valve with a 20- μ l sample loop with auto-sampler was used to inject the samples. Column A was outside the main system and perfused by mobile phase at 0.1 ml/min, with a Waters HPLC pump. At sample injection, the flow rate was raised to 0.25 ml/min, and 45 s later the small column was connected to main column B for a minute.

Column A was then isolated and perfused with 50% buffer and 50% acetonitrile for 10 min, and eluted with the mobile phase for a further 5 min. The flow rate of column A was then decreased to 0.1 ml/min, prior to the next sample injection.

Drugs

ASE (Org 5222; *trans*-5-chloro-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7,5]-oxepino-[4,5c]pyrrolidine maleate; Organon, Lanarkshire, UK) and the 5-HT_{1A} blocker WAY100635 (Wyeth, Philadelphia, PA) were dissolved in deionized water. Vehicle or drug was administered subcutaneously through the implanted catheter in a volume of 1 ml/kg. WAY100635 was given 30 min prior to ASE. The doses of ASE were selected based on published *in vivo* behavioral and receptor studies (De Boer *et al*, 1993; Schotte *et al*, 1996).

Data Analysis

All dialysis probe placements were checked after killing the rats. Only results derived from rats with correctly positioned probes were included in the data analysis. Three rats, which were sedated following surgery, were not studied. The mean value of three consecutive stable samples prior to drug injection was set at 100% and considered the predrug basal level. Basal extracellular levels of each neurotransmitter in the mPFC, HIP, and NAC were compared by one-way analysis of variance (ANOVA). The time-dependent effect of drugs on each neurotransmitter in the three regions was analyzed using a repeated-measure ANOVA, with treatment group as a fixed factor and time as the within-subject factor. The response area under the curve (AUC) was calculated by the trapezoid rule, using data following injection of the vehicle or drug. Two-way ANOVA was used to compare the effect of treatment on AUC for each neurotransmitter across the three regions. ANOVA was followed by the least-square significant difference *post hoc* pairwise comparison procedure. The level of significance was set at $p < 0.05$. All analyses were performed using SASTM (SAS Institute Inc., Cary, NC, USA) statistical software.

RESULTS

Baseline Extracellular DA, NE, Serotonin, ACh, Glutamate, and GABA Levels in the Cortex, HIP, and NAC

One-way ANOVA showed no significant differences among the treatment groups regarding baseline extracellular DA, ACh, NE, 5-HT, glutamate, and GABA levels in the three brain regions studied. Baseline extracellular DA levels in all rats in this study were 0.12 ± 0.02 nM in the mPFC ($F = 1.16$, $p = 0.36$, $n = 38$), 0.15 ± 0.03 nM in the HIP ($F = 2.70$, $p = 0.08$, $n = 38$), and 0.75 ± 0.06 nM in the NAC ($F = 2.24$, $p = 0.16$, $n = 20$). Baseline NE levels were 0.19 ± 0.03 nM in the mPFC ($F = 0.84$, $p = 0.46$, $n = 20$) and 0.12 ± 0.02 nM in the HIP ($F = 2.64$, $p = 0.09$, $n = 19$). Baseline 5-HT levels in the dialysates were 0.12 ± 0.01 nM in the mPFC ($F = 2.32$, $p = 0.18$, $n = 15$) and 0.13 ± 0.02 nM in the HIP ($F = 1.77$, $p = 0.24$, $n = 15$). Baseline ACh levels were 0.58 ± 0.14 nM in

the mPFC ($F = 2.77$, $p = 0.08$, $n = 28$), 0.47 ± 0.09 nM in the HIP ($F = 3.00$, $p = 0.10$, $n = 23$), and 0.44 ± 0.17 nM in the NAc ($F = 1.76$, $p = 0.24$, $n = 21$). Baseline extracellular glutamate levels were 2.38 ± 0.60 μ M in the mPFC ($F = 0.85$, $p = 0.40$) and 2.46 ± 0.67 μ M in the NAc ($F = 1.69$, $p = 0.26$, both $n = 10$). Baseline GABA levels were 0.04 ± 0.01 μ M in the mPFC ($F = 0.57$, $p = 0.48$) and 0.05 ± 0.01 μ M in the NAc ($F = 0.35$, $p = 0.58$, both $n = 10$). Vehicle administration did not affect extracellular baseline levels of these neurotransmitters, in these regions.

Effect of ASE on DA Efflux in the MPFC, HIP, and NAc

ASE increased extracellular DA in the mPFC, HIP, and NAc (Figure 1). Repeated-measure ANOVA of mPFC data showed significant overall group ($F = 7.31$, $p = 0.0001$) and time effects ($F = 2.66$, $p = 0.02$). The group $\times$ time effect was not significant. *Post hoc* analysis revealed that ASE at 0.05 mg/kg ($p = 0.004$), ASE at 0.1 mg/kg ($p = 0.005$), and ASE at 0.5 mg/kg ($p = 0.0003$) were significantly different from the vehicle, whereas ASE at 0.01 mg/kg differed from the higher doses but not the vehicle. For the HIP, both overall group ($F = 4.32$, $p = 0.01$) and time ($F = 2.40$, $p = 0.03$) effects were significant, but the group $\times$ time interaction was not. *Post*

hoc analysis showed that ASE at 0.05 mg/kg ($p = 0.03$) and ASE at 0.5 mg/kg ($p = 0.004$) differed significantly from the vehicle; ASE at 0.1 mg/kg differed at the trend level ($p = 0.09$); ASE at 0.01 mg/kg did not differ from the vehicle, but was significantly different from the other doses studied. For the NAc, the group $\times$ time interaction was significant ($F = 1.84$, $p = 0.03$). The time effect was not significant, but the overall group effect was ($F = 5.93$, $p = 0.01$). *Post hoc* analysis revealed that ASE at 0.5 mg/kg was significantly different from the vehicle ($p = 0.002$), ASE at 0.1 mg/kg ($p = 0.005$), and ASE at 0.05 mg/kg ($p = 0.01$). The response AUC (Figure 1) was significantly different among the treatment groups for each region, mPFC ($F = 6.99$, $p = 0.001$), HIP ($F = 4.69$, $p = 0.007$), and NAc ($F = 5.70$, $p = 0.01$). *Post hoc* analysis showed essentially the same group differences within region as the repeated-measure ANOVA reported above. *P*-values for differences among the response AUCs are given in the legend to Figure 1.

Two-way ANOVA was used to compare the treatment group across the three regions. The interaction of region and treatment was not significant. The main effects of region, regardless of dose ($F = 3.20$, $p = 0.05$), and treatment group, regardless of region ($F = 15.35$, $p = 0.0001$), were significant. ASE at 0.05 mg/kg ($p = 0.03$) and ASE at 0.1 mg/kg

Figure 1 Effect of ASE (0.01, 0.05, 0.1, and 0.5 mg/kg, s.c.) on DA efflux in rat mPFC, HIP, and NAc. Data are mean $\pm$ SEM of the dialysate DA levels, expressed as a percentage of the baseline. The response AUC (RAUC) was significantly different among the treatment groups for each region: mPFC ($F = 6.99$, $p = 0.001$), HIP ($F = 4.69$, $p = 0.007$), and NAc ($F = 5.70$, $p = 0.01$). For the mPFC, *post hoc* analysis revealed that the ASE 0.05 mg/kg ($p = 0.004$), and ASE 0.5 mg/kg ($p = 0.0003$) groups were significantly different from vehicle. Also, ASE at 0.1 mg/kg, was significantly different from ASE at 0.5 mg/kg ($p = 0.0003$). For the HIP, both ASE at 0.05 mg/kg ($p = 0.01$) and ASE at 0.5 mg/kg ($p = 0.003$) were significantly different from the vehicle and ASE at 0.01 mg/kg ($p = 0.02$, $p = 0.003$), respectively. For the NAc, ASE at 0.5 mg/kg was significantly different from the vehicle group ($p = 0.002$), ASE at 0.05 mg/kg ($p = 0.01$), and ASE at 0.1 mg/kg ($p = 0.01$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, vs vehicle; # $p < 0.05$, ## $p < 0.01$, vs ASE at 0.01 mg/kg; and & $p < 0.05$, vs ASE at 0.05 mg/kg. VEH, vehicle.

kg ($p = 0.02$), but not ASE at 0.5 mg/kg ($p = 0.09$), produced greater increases on DA efflux in the mPFC compared with in the NAC; there were no differences between the effects of ASE on DA efflux in the mPFC and HIP at any of the doses studied.

Effects of ASE on ACh Efflux in the mPFC, HIP, and NAC

ASE increased extracellular ACh in the mPFC and HIP but not in the NAC (Figure 2). Repeated-measure ANOVA of mPFC ACh data showed significant overall group ($F = 7.94$, $p = 0.0004$) and time effects ($F = 2.43$, $p = 0.03$). The group $\times$ time effect was not significant. *Post hoc* analysis revealed that ASE at 0.05 mg/kg ($p = 0.04$), ASE at 0.1 mg/kg ($p = 0.00$), and ASE at 0.5 mg/kg ($p = 0.002$) were significantly different from the vehicle, whereas ASE at 0.01 mg/kg differed from the higher doses, but not the vehicle. For the HIP, both the overall group ($F = 3.43$, $p = 0.03$) and time ($F = 3.44$, $p = 0.004$) effects were significant, but the group-time interaction was not. *Post-hoc* analysis showed that ASE at 0.5 mg/kg differed significantly from the vehicle ($p = 0.005$), from ASE at 0.01 mg/kg ($p = 0.01$), and ASE at 0.05 mg/kg ($p = 0.04$). The response AUC (Figure 2) was significantly different among the treatment groups for each region, mPFC ($F = 7.02$, $p = 0.001$) and HIP ($F = 3.99$,

$p = 0.02$) but not the NAC. *Post hoc* analysis showed essentially the same group differences within region as the repeated-measure ANOVA reported above. *P*-values for differences among the response AUCs within regions are given in the legend to Figure 2.

Two-way ANOVA was used to compare the treatment groups in the mPFC and HIP. The interaction of region and treatment was not significant. The main effect of group (dose), was significant for both regions ($F = 10.08$, $p = 0.0001$). There was no significant effect for region at any of the doses studied.

Effect of Pretreatment with WAY100635 on ASE-Induced DA and ACh Release in the mPFC and HIP

WAY100635 (0.2 mg/kg, s.c.) alone had no significant effect on DA or ACh efflux in the mPFC or the HIP (Figure 3). Pretreatment with WAY100635 significantly attenuated the increased DA efflux induced by ASE at 0.1 mg/kg in both the mPFC ($F_{1,9} = 20.56$, $P < 0.001$) and HIP ($F_{1,9} = 8.47$, $p = 0.005$). WAY100635 also significantly inhibited the effect of ASE on ACh efflux in the mPFC ($F_{1,9} = 16.58$, $P < 0.001$).

Figure 2 Effect of ASE (0.01, 0.05, 0.1, and 0.5 mg/kg, s.c.) on ACh efflux in the rat mPFC, HIP, and NAC. Data are means $\pm$ SEM of the dialysate ACh levels, expressed as percentage of the baseline. The response AUC (RAUC) was significantly different among the treatment groups: mPFC ($F = 7.02$, $p = 0.001$), HIP ($F = 3.99$, $p = 0.02$), but not the NAC. For the mPFC, *post hoc* analysis revealed that the vehicle group was significantly different from ASE 0.1 mg/kg ($p = 0.003$) and ASE 0.5 mg/kg ($p = 0.002$) groups. Also, ASE at 0.01 mg/kg was significantly different from ASE at 0.05 mg/kg ($p = 0.04$), ASE at 0.1 mg/kg ($p = 0.001$), and ASE at 0.5 mg/kg ($p = 0.001$). For the HIP, ASE at 0.5 mg/kg was significantly different from the vehicle group ($p = 0.004$), and ASE at 0.5 mg/kg was different from ASE at 0.01 mg/kg ($p = 0.01$) and ASE at 0.5 mg/kg ($p = 0.01$). ** $p < 0.01$, vs vehicle; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$, vs ASE 0.01 mg/kg dose; and * $p < 0.05$, vs ASE 0.05 mg/kg dose. VEH, vehicle.

Figure 3 Effect of pretreatment with WAY100635 (0.2 mg/kg, s.c.) on ASE (0.1 mg/kg, SC)-induced DA and ACh efflux in rat mPFC and HIP. Data are means $\pm$ SEM of the dialysate DA levels, expressed as percentage of the baseline. Pretreatment with WAY100635 significantly blocked increased DA efflux induced by ASE at 0.1 mg/kg in both regions, and blocked ACh efflux in the mPFC. VEH, vehicle.

Effect of ASE on NE Efflux in the mPFC and HIP

ASE increased extracellular NE in the mPFC and HIP (Figure 4). Repeated-measure ANOVA of mPFC data showed significant overall group effect ($F = 5.08$, $p = 0.01$); the time effect was not significant. The group $\times$ time effect was not significant. *Post hoc* analysis of mPFC data revealed that ASE at 0.1 mg/kg was significantly different from the vehicle ($p = 0.001$), ASE at 0.01 mg/kg ($p = 0.01$), and ASE at 0.05 mg/kg ($p = 0.01$). For the HIP, both overall group ($F = 4.60$, $p = 0.02$) and time ($F = 2.40$, $p = 0.03$) effects were significant, but the group $\times$ time interaction was not. *Post hoc* analysis revealed that ASE at 0.1 mg/kg was significantly different from the vehicle ($p = 0.01$) and ASE at 0.01 mg/kg ($p = 0.01$). The response AUC (Figure 4) was significantly different among the treatment groups for each region, mPFC ($F = 5.69$, $p = 0.01$) and HIP ($F = 4.45$, $p = 0.02$). *Post hoc* analysis results showed the same group differences within region as the repeated-measure ANOVA reported above. *P*-values for differences among the response AUCs are given in the legend to Figure 4.

Two-way ANOVA was used to compare the treatment group across the three regions. The interaction of region and treatment was not significant. The main effects of region, regardless of dose ($F = 3.20$, $p = 0.05$), and treatment group, regardless of region ($F = 15.35$, $p = 0.0001$), were significant. ASE at 0.05 mg/kg ($p = 0.03$) and ASE at 0.1 mg/kg ($p = 0.02$), but not ASE at 0.5 mg/kg ($p = 0.09$), produced greater increases on DA efflux in the mPFC compared with in the NAc; there were no differences between the effects of

ASE on DA efflux in the mPFC and HIP at any of the doses studied.

Effect of ASE on Serotonin Efflux in the mPFC and HIP

As shown in Figure 5, ASE, at 0.05 and 0.1 mg/kg, had no effect on 5-HT efflux in either the mPFC or HIP.

Effect of ASE on Glutamate and GABA Efflux in the mPFC and NAc

ASE at 0.1 mg/kg had no significant effect on glutamate or GABA efflux in either the mPFC or NAc (data not shown).

DISCUSSION

The main findings of this study are the following: (1) ASE produced a dose-dependent increase in DA efflux in the mPFC and HIP; (2) ASE, at 0.5 mg/kg, but not at lower doses increased DA efflux in the NAc; (3) ASE dose dependently increased ACh efflux in the mPFC and HIP, but not in the NAc; (4) the effect of ASE on DA efflux in the mPFC and HIP was blocked by pretreatment with the 5-HT_{1A} antagonist/D₄ agonist, WAY100635; (5) ASE increased NE efflux in the mPFC and HIP, but had no effect on 5-HT efflux in either region, at the doses tested; and (6) ASE, at the same dose that significantly increased DA and NE efflux in the mPFC and HIP, had no effect on glutamate and GABA efflux in the mPFC or NAc. These results suggest that acute

Figure 4 Effect of ASE (0.01, 0.05, and 0.1 mg/kg, s.c.) on NE efflux in the rat mPFC and HIP. Data are means $\pm$ SEM of the dialysate NE levels, expressed as percentage of baseline. The response AUC (RAUC) was significantly different among the treatment groups for MPFC ($F = 5.69$, $p = 0.01$) and HIP ($F = 4.45$, $p = 0.02$). For the mPFC, *post hoc* analysis revealed that the vehicle group was significantly different from the ASE 0.1 mg/kg ($p = 0.001$) group. Also, ASE at 0.1 mg/kg was significantly different from ASE at 0.01 mg/kg ($p = 0.01$) and ASE at 0.05 mg/kg ($p = 0.01$). For the HIP, the ASE 0.1 mg/kg group was significantly different from the vehicle group ($p = 0.01$). Also, ASE at 0.1 mg/kg was significantly different from ASE at 0.01 mg/kg ($p = 0.01$). ** $p < 0.01$, vs vehicle; # $p < 0.05$, vs ASE 0.01 mg/kg dose; and & $p < 0.05$, vs ASE 0.05 mg/kg dose. VEH, vehicle.

Figure 5 Effect of ASE (0.05 and 0.1 mg/kg, s.c.) on serotonin (5-HT) efflux in the rat mPFC and HIP. Data are means $\pm$ SEM of the dialysate 5-HT levels, expressed as percentage of baseline. ASE at 0.05 and 0.1 mg/kg had no effect on 5-HT efflux in either region. VEH, vehicle.

treatment with ASE has effects on the mPFC, HIP, and NAc DA, NE, and ACh efflux comparable to those of clozapine and other atypical antipsychotic drugs, which are relatively more potent 5-HT_{2A} antagonists than D₂ antagonists.

DA Efflux

Atypical antipsychotics have been reported to preferentially increase DA efflux in the mPFC and HIP compared with in

the NAc in freely moving rats (Chung *et al*, 2004; Ichikawa *et al*, 2001; Kuroki *et al*, 1999), compared with typical antipsychotic drugs. ASE, in this study, at doses less than 0.5 mg/kg, also showed preferential increase in cortical and HIP DA efflux compared with in the NAc. This action is due, in part, to blockade of 5-HT_{2A} receptors in the cerebral cortex and relatively weaker or negligible occupation of D₂ and D₁ receptors (Matsubara *et al*, 1993), since it can be mimicked by the combination of potent 5-HT_{2A} antagonism

and weak blockade of D₂ receptors (Liegeois *et al*, 2002; Bonaccorso *et al*, 2002; Li *et al*, 2005). As reported by Schotte *et al* (1996), these agents, including clozapine, risperidone, 9-hydroxyrisperidone (paliperidone), olanzapine, pipamperone, quetiapine, sertindole, ziprasidone, and zotepine, also have higher occupancy in the rat cortex and striatum of 5-HT_{2A} than D₂ receptors, respectively, at all but the highest doses studied. PET studies are also consistent with the view that at clinically relevant doses, those agents, which are direct acting antagonists of D₂ receptors, have higher occupancy of D₂ than 5-HT_{2A} receptors (Goyer *et al*, 1996; Nyberg *et al*, 1999; Gefvert *et al*, 2001; Kessler *et al*, 2005). Aripiprazole, a partial D₂ receptor agonist, has a higher D₂ than 5-HT_{2A} receptor occupancy at clinical doses, but, nevertheless, has a weak functional effect to inhibit D₂ receptor stimulation (Mamo *et al*, 2007). As mentioned under Introduction, diminished dopaminergic and noradrenergic function in the cortex and HIP have been implicated in the pathophysiology of cognitive impairment, negative symptoms, and perhaps depression in patients with schizophrenia (Meltzer and McGurk, 1999; Millan, 2006; Juckel *et al*, 2006). Recent studies have clearly demonstrated the importance of increased cortical D₁ receptor stimulation for specific types of memory, including working memory and social memory (Castner and Williams, 2007; Nagai *et al*, 2007), possibly by increasing the activity of pyramidal neurons, modulation of glutamate NMDA receptor signaling at critical nodes within local circuits and distributed networks (Castner and Williams, 2007), fine tuning GABAergic interneurons (Kroner *et al*, 2007), and enhancing the release of cortical ACh (Di Cara *et al*, 2007). Thus, these effects on the efflux of cortical DA and NE may be clinically relevant to the treatment of schizophrenia (Ichikawa *et al*, 2001; Meltzer *et al*, 2003; Arnsten and Li, 2005), as well as to the ability of these same agents, in combination with at least some antidepressant drugs, to be effective in patients with major depression who fail to respond to antidepressant drug treatment alone (Shelton *et al*, 2001).

The increased efflux of DA in the mPFC and HIP produced by antipsychotic drugs (eg, aripiprazole, clozapine, olanzapine, risperidone, ziprasidone) has been shown to be partially or completely (eg, quetiapine) blocked by the 5-HT_{1A} antagonist/D₄ receptor agonist WAY100635 in most (Ago *et al*, 2005; Chung *et al*, 2004; Ichikawa *et al*, 2001; Ichikawa and Meltzer, 2000; Li *et al*, 2004; Sprouse *et al*, 1999; Yoshino *et al*, 2004), but not all (Assie *et al*, 2005), studies. Although the recent demonstration of relatively potent D₄ agonist activity of WAY100635 (Chemel *et al*, 2006) complicates the interpretation of studies with this agent, the role of 5-HT_{1A} receptor agonism in mediating the effects of atypical antipsychotics on mPFC DA efflux is also supported by experiments in which the highly selective 5-HT_{1A} agonist, BAY × 3702 (BAY; 10–40 µg/kg, i.v.), increased DA release in the mPFC. WAY100635 reversed the effects of BAY in both the areas. The atypical antipsychotics, clozapine, olanzapine, and ziprasidone (but not haloperidol), enhanced DA release in the mPFC of wild-type, but not 5-HT_{1A}-knockout, mice after systemic and local (clozapine and olanzapine) administration in the mPFC (Diaz-Mataix *et al*, 2005). Local administration of WAY100635 into the mPFC of male rats blocks the effect of

systemic clozapine on cortical DA release (Li *et al*, unpublished data). ASE is similar to quetiapine (Ichikawa *et al*, 2002c) in that its ability to increase the efflux of cortical DA in rats was completely blocked by WAY100635. The clinical significance, if any, of this difference is unknown at present. The increase in cortical DA release produced by local injection of clozapine or olanzapine into the cortex was also abolished in 5-HT_{1A}-knockout mice (Diaz-Mataix *et al*, 2005). Partial agonism at the 5-HT_{1A} receptor has been reported for some (eg, clozapine, quetiapine, ziprasidone), but not all, atypical antipsychotic drugs (eg, olanzapine and risperidone) (Meltzer *et al*, 2003), a possible explanation for the blockade of the antipsychotic-induced DA efflux by WAY100635. Unlike clozapine, quetiapine, and ziprasidone (Meltzer *et al*, 2003), ASE does not behave like a 5-HT_{1A} agonist in cloned cell preparations (Shahid *et al*, 2007), nor does it enhance cortical 5-HT efflux at the doses tested. Thus, the mechanism by which ASE influences 5-HT_{1A}-receptor stimulation requires further investigation.

ASE significantly increased DA efflux in the NAc at the highest dose studied (0.5 mg/kg), but not at 0.05 mg/kg, which increased DA efflux in the mPFC and HIP. Atypical antipsychotic drugs, including clozapine and olanzapine, can increase DA efflux in the NAc, although the magnitude of the effect is significantly smaller than that produced in the mPFC (Ichikawa *et al*, 2002c; Kuroki *et al*, 1999; Shilliam and Dawson, 2005). Shilliam and Dawson (2005) demonstrated that this increase was confined to the shell of the NAc and did not occur in the NAc core. We have previously shown that 5-HT_{2C}-receptor antagonism, in combination with D₂-receptor antagonism, may contribute to the ability of these agents to enhance DA release in the NAc (Bonaccorso *et al*, 2002; Li *et al*, 2005). The potent 5-HT_{2C} antagonist, SB242084, significantly potentiated low-dose haloperidol-induced increase in DA release in the NAc (Li *et al*, 2005). The selective 5-HT_{2C} antagonists, SB206553 and SB242084, alone have been shown to increase DA efflux in both the mPFC and NAc (De Deurwaerdere and Spampinato, 2001; Di Matteo *et al*, 2001, 1998, 1999, 2000; Millan *et al*, 1998, 2003). As ASE is a very potent 5-HT_{2C} antagonist (pK_i 10.46 ± 0.15; Shahid *et al*, 2007; Prinssen *et al*, 2000), this may, at least in part, be involved in mediating its effects in the NAc. Occupancy of D₂ receptors may also contribute to the ability of ASE to enhance DA efflux in the NAc (Kuroki *et al*, 1999). There is also some evidence that the increase in DA efflux produced by the atypical antipsychotic drugs is mediated, in part, by α_{2A}-adrenergic-receptor blockade (Blake *et al*, 1998; Bymaster *et al*, 1996; Gobert *et al*, 1998; Hertel *et al*, 1999; Millan *et al*, 2000; Wadenberg *et al*, 2007), which may also contribute to the action of ASE in this regard, as it also an α_{2A}-adrenergic-receptor antagonist (Shahid *et al*, 2007).

ACh Efflux

ASE preferentially increased ACh efflux in both the mPFC and HIP, but not in the NAc, which is also the case for clozapine, olanzapine, risperidone, quetiapine, and ziprasidone (Ichikawa *et al*, 2002a, b, c; Parada *et al*, 1997; Shirazi-Southall *et al*, 2002). Muscarinic cholinergic receptors are unlikely to be important for this action, since, unlike

clozapine or olanzapine, ASE has no appreciable affinity for these receptors (Shahid *et al*, 2007). Indirect 5-HT_{1A}-receptor stimulation seems more likely to be important for this effect, since the increase in ACh induced by ASE is sensitive to blockade by WAY100635. This is similar to the profile of quetiapine (Ichikawa *et al*, 2002c). It has recently been reported that systemic administration of risperidone, at 1 and 2 mg/kg, dose dependently increased ACh efflux in the rat mPFC; this increase was antagonized by systemic administration of high (1 and 3 mg/kg), but not by a lower dose (0.1 mg/kg) of WAY100635, which preferentially blocks presynaptic 5-HT_{1A} autoreceptors (Sato *et al*, 2007; Ago *et al*, 2003). Local application of WAY100635 into the mPFC did not affect ACh release in the mPFC, but did attenuate risperidone-induced increases in ACh efflux (Sato *et al*, 2007). Local application of neither risperidone (3 and 10 μ M) and the 5-HT_{1A}-receptor agonist, L-750,667 (8-hydroxy-2-(di-n-propylamino)tetralin; 1 and 10 μ M), nor the DA D₄-receptor antagonist, 3-(4-(4-iodophenyl)piperazine-1-yl)methyl-1H-pyrrolo[2,3-b]pyridine (1 and 10 μ M), into the mPFC affected ACh release in the mPFC (Sato *et al*, 2007). Taken together, these results suggest that atypical antipsychotic drugs increase ACh efflux in the mPFC through a circuit, which includes prefrontal 5-HT_{1A}-receptor activation. Histamine H₁ and α_2 -adrenoceptor blockade have also been shown to enhance ACh release (Dringenberg *et al*, 1998; Tellez *et al*, 1997), and, thus, may be also involved in ASE-induced ACh release in both mPFC and HIP.

NE and Serotonin Release

ASE, like other atypical antipsychotics, dose dependently increased NE release in the rat mPFC and HIP (Westerink *et al*, 1998). ASE increased DA efflux in the mPFC at 0.05 mg/kg, but not NE. The increases in DA and NE efflux in the mPFC at 0.1 mg/kg were comparable. The effect on DA and NE efflux in the HIP were more similar. Increased NE efflux, rather than, or in addition to, DA efflux, may be important for the ability of atypical antipsychotic drugs to improve cognitive function in schizophrenia (Arnsten and Li, 2005; Rossetti and Carboni, 2005). Increases in NE and DA release in the rat mPFC have been reported with 5-HT_{1A}-receptor agonists (Hajos-Korcsok *et al*, 1999; Owen and Whitton, 2003); the α_2 -adrenoceptor antagonists RS79948 (Devoto *et al*, 2004) and 1-(2-pyrimidinyl)piperazine (Gobert *et al*, 1999); and the selective 5-HT_{2C} antagonist, SB242084 (Millan *et al*, 1998). As previously noted, ASE is a potent 5-HT_{2C}-receptor and α_2 -adrenoceptor antagonist (Schotte *et al*, 1996; Shahid *et al*, 2007). A combination of these actions may mediate its ability to increase NE and DA efflux in the mPFC and HIP.

ASE did not affect 5-HT efflux in either the mPFC or HIP at the doses tested, despite its α_2 -adrenoceptor-antagonist properties. The α_2 -adrenoceptor antagonist, yohimbine, increased, and the α_2 -agonist, clonidine, decreased, the extracellular levels of 5-HT in the rat frontal cortex (Cheng *et al*, 1993). One possible explanation for the lack of effect of ASE on 5-HT efflux is that the degree of α_2 -adrenoceptor block was insufficient at the doses used in the current study, but this seems unlikely in that its affinity for the α_2 -adrenoceptor is identical to that for the D_{2L} receptor. Alternatively, given the complex multireceptor profile of

atypical antipsychotic drugs, the inability of ASE to elevate cortical 5-HT levels may result from action, which negates the effects of α_2 -adrenoceptor blockade. Indeed, risperidone has been reported in several studies to increase mPFC 5-HT release (Cartmell *et al*, 2001; Hertel *et al*, 1996; Ichikawa *et al*, 1998), whereas clozapine and olanzapine do not. The lack of an increase in 5-HT efflux in the mPFC by ASE suggests that stimulation of 5-HT_{1A} receptors, which appears to be critical for its ability to enhance ACh and DA efflux, is not due to 5-HT efflux in this region. This is consistent with the evidence that local administration of the 5-HT_{1A} agonist, 8-OH-DPAT, did not enhance ACh efflux in the mPFC (Sato *et al*, 2007).

Glutamate and GABA Efflux

In the present study, ASE did not alter extracellular glutamate and GABA levels in the mPFC, at a dose that did enhance DA, NE, and ACh efflux in that region. It also had no effect on glutamate or GABA efflux in the NAc. In this regard, it differs from clozapine, which significantly increased glutamate efflux in the rat mPFC (Daly and Moghaddam, 1993; Yamamoto and Cooperman, 1994). It also differs from clozapine and haloperidol, which decreased basal GABA release in the mPFC (Bourdelaïs and Deutch, 1994). These data indicate that modulation of cortical glutamate and GABA efflux does not represent a common action of antipsychotic drugs. The significance of these differences between antipsychotic drugs remains to be determined.

Both risperidone and clozapine have been reported to diminish GABA release in the globus pallidus (Grimm and See, 1998). Clozapine has also been shown to dose dependently block phencyclidine-induced acute increases in glutamate efflux in the rat mPFC, as well as to block PCP-induced hyperlocomotion. Clozapine also attenuated acute increases in glutamate efflux in the mPFC induced by local perfusion with the competitive NMDA-receptor antagonist, CPP (Abekawa *et al*, 2006). Further study of a broader dose range of ASE is required to assess the effects of ASE on glutamatergic mechanisms.

Conclusions

In conclusion, acute administration of ASE significantly increased cortical and hippocampal DA, NE, and ACh efflux in a dose-dependent and regionally selective manner, but had no effect on cortical and HIP 5-HT, glutamate, or GABA efflux. The effects on DA, NE, and ACh efflux are comparable to those previously reported with clozapine and quetiapine. Several key differences between clozapine and ASE were noted, including the ability of WAY100635 to block ACh efflux and the lack of an effect on cortical GABA and glutamate efflux. Overall, these results suggest ASE is an antipsychotic, which should improve cognition and negative symptoms in schizophrenia. Further study of its efficacy and side effect profile is clearly of interest.

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DISCLOSURE/CONFLICT OF INTEREST

HYM has been a consultant to, or a grantee, or both, of the following pharmaceutical companies within the past 3 years: Abbott, ACADIA, ARYx, Astra Zeneca, BioLine Rx, Bristol Meyers Squibb, Cephalon, Eli Lilly, GSK, Janssen Pharmaceuticals, Minster, Organon Laboratories Limited, Ovation, Pfizer, SK, Solvay and Takeda. Mohammed Shahid is an employee and shareholder of Organon Laboratories Limited. Erik Wong was an employee of Pfizer Inc. during the period of his work on this research, and is a shareholder of Pfizer. Erik Wong is currently an employee of Astra Zeneca. The author(s) declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional services, and they have no personal financial holdings that could be perceived as constituting a potential conflict of interest, with the exception that HYM is a stockholder of ACADIA, which has no direct or indirect involvement in this study.

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