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Title Page

The dopamine stabilizers (-)-OSU6162 and ACR16 show high in vivo D_2 receptor occupancy, antipsychotic-like efficacy and low potential for motor side effects in the rat

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Running Title Page

Running Title: Pharmacology of the dopamine stabilizers (-)-OSU6162 & ACR16

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Abbreviations: (-)-OSU6162, (S)-(-)-3-methylsulfonylphenyl-1-propylpiperidine; ACR16, (4-(3-methylsulphonyl-phenyl-N-n-propylpiperidine); CAR, conditioned avoidance response; NSD 1015HCl, 3-hydroxybenzyl hydrazine hydrochloride; HPLC-ED, high pressure liquid chromatography with electrochemical detection

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Abstract

"Dopamine stabilizers" is a new class of compounds which have the ability to reverse both hypo as well as hyperdopaminergia in vivo. This class, exemplified by the phenylpiperidines (-)-OSU6162 & ACR16, although lacking high in vitro binding affinity for dopamine D_2 receptor ((-)-OSU6162:Ki 447nM, ACR16:Ki >1μM) show functional actions suggestive of their interaction. Hence we evaluated in vivo D_2 occupancy of these agents in rats and correlated it to observed effects in a series of behavioral, neurochemical and endocrine models relevant to the dopamine system and antipsychotic effect. Both (-)-OSU6162 and ACR16 showed robust dose dependent striatal D_2 occupancy with ED_{50} values of 5.27 and 18.99 mg/kg, s.c., respectively, and functional assays showed no partial agonism. Over an occupancy range of 37–87% (3–60 mg/kg) for (-)-OSU6162 and 35–74% (10–60 mg/kg) for ACR16, we observed both inhibitory (amphetamine induced locomotor activity) and stimulatory effects (in habituated rats). Haloperidol, over a similar occupancy range (33–78%), potently inhibited psychostimulant activity and induced catalepsy, but failed to activate habituated animals. In the conditioned avoidance response (CAR) assay, ACR16 was clearly more efficacious than (-)-OSU6162. Also both these compounds demonstrated significant preferential Fos induction in the nucleus accumbens as compared to the dorsolateral striatum, a strong predictor of atypical antipsychotic efficacy. The results suggest that dopamine stabilizers exhibit locomotor stabilizing as well as antipsychotic-like effects with low motor side-effect liability, in a dose range that corresponds to high D_2 in vivo occupancy.

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Introduction

Dysregulation or disruption of dopaminergic function underlies a variety of neuropsychiatric disorders including schizophrenia and Parkinson's disease. Schizophrenia is thought to result from subcortical hyperdopaminergia (leading to positive symptoms) and cortical hypodopaminergia (leading to negative and cognitive deficits) (Abi-Dargham, 2004). Efforts to treat symptoms of schizophrenia have focused on blocking dopaminergic activity and while the introduction of antipsychotics have revolutionized the treatment of this debilitating disorder, there remains an unmet need to treat negative symptoms and cognitive deficits (Barbeau, 1961; Seeman, 1987; Nieoullon and Coquerel, 2003; Carlsson et al., 2004). Thus, optimal treatment for these conditions may not rely solely on dopamine blockade, but may include a dopamine tone-dependent modulation or "stabilization" of the dopaminergic system. There is now a class of drugs that has been suggested to possess such properties, exemplified by (-)-OSU6162 and ACR16 (Sonesson et al., 1994; Ekesbo et al., 1997; Tedroff et al., 1998; Nichols et al., 2002; Carlsson et al., 2004; Nilsson et al., 2004; Rung et al., 2005). These compounds are reported to lack high in vitro affinity for various neuroreceptors. For (-)-OSU6162, D_2 and D_3 Ki's were 447 and 1305 nM, respectively and were reported with Ki's >1 µM for other targets (Sonesson et al., 1994), while for ACR16 the Ki's for a number of targets, including monoaminergic receptors, were reported to be >10 µM (Petterson et al., 2002). Similar results were found by researchers at Eli Lilly with D_2/D_3 Ki's of 23/2.2 µM and 0.9/2.8 µM for ACR16 and (-)-OSU6162, respectively using 7-OH-DPAT as radioligand in cloned cells transfected with human D_2/D_3 receptors (Dr Sheila Little, personal communication).

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(-)-OSU6162 (also called PNU-96391A) ((S)-(-)-3-methylsulfonylphenyl-1-propylpiperidine) was identified amongst a series of substituted (S)-phenylpiperidines examined for their ability to interact with central dopamine receptors, and it was found to be highly active in vivo on the synthesis and turnover of dopamine (Sonesson et al., 1994). It also caused inhibition of amphetamine induced hyperlocomotion and stimulation of locomotor activity in rats with low psychomotor activity without causing motor impairment or catalepsy (Svensson et al., 1993; Waters et al., 1993; Sonesson et al., 1994). This class of compounds have been named "dopamine stabilizers" based on their ability to either stimulate, suppress or show no effect on motor activity, depending upon the prevailing dopaminergic tone (Carlsson et al., 2004). In search for compounds through a similar in vivo testing strategy, a close structural analog ACR16 (4-(3-methylsulphonyl-phenyl-N-n-propylpiperidine) emerged as a molecule displaying a similar pharmacological profile as that of (-)-OSU6162. It induced a dose-dependent increase in the dopamine metabolite DOPAC in the striatum, cortex and limbic areas, while exploratory locomotor activity was unaffected. Also, it increased locomotor activity in habituated animals, while antagonizing amphetamine, MK-801 and cocaine-induced locomotor activity in mice and recently it has been shown to reverse social withdrawal induced by MK-801 in rats (Carlsson, 2002; Waters et al., 2002; Nilsson et al., 2004).

While these drugs show intriguing abilities to modify dopamine-related behaviors in a bi-directional manner, the mechanisms by which these effects are induced are not

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completely understood. Although these compounds show weak in vitro D_2 receptor affinities (see above), a PET study in the rhesus monkey showed that continuous intravenous administration of (-)-OSU6162 produced nearly 80% displacement of [^{11}C]raclopride in the striatal brain area (Neu et al., 1997; Tedroff et al., 1998; Ekesbo et al., 1999). So our first objective in this study was to evaluate the in vivo rat striatal dopamine D_2 receptor occupancy and catalepsy induction by (-)-OSU6162 and ACR16, over their effective dose ranges, using previously validated methods (Wadenberg et al., 2000). Secondly, we examined the ability of these compounds to stabilize motor function in animals made hyperactive by means of d-amphetamine pretreatment and also examined whether the compounds could affect motor activity in rats with low baseline activity as the result of repeated habituation to the activity cages. To examine if stabilization could be obtained with a standard dopamine D_2 blocker, we included haloperidol at doses that caused equivalent D_2 occupancy as those induced by (-)-OSU6162 and ACR16. To further characterize the functional effects of these drugs we examined if ACR16 and (-)-OSU6162 would show partial agonist activity in vivo at the dopamine D_2 autoreceptors and also examined if they could increase plasma levels of prolactin. Finally, the ability of these compounds to inhibit conditioned avoidance responding (CAR) (Wadenberg et al., 2000) and to induce expression of Fos in the nucleus accumbens (Deutch et al., 1992; Robertson et al., 1994) were used as additional assessments of their antipsychotic potential.

Methods

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Animals. The experiments were carried out on adult male Sprague-Dawley rats, weighing 250-275g when procured from Charles River, Montreal, Canada. They were housed two per cage on a 12:12 reverse light/dark cycle (lights off at 8.00_{AM}) with free access to food and water. The animals were allowed to acclimatize for a minimum of five days before being used for experimentation. All experiments were approved by the institution's Animal Care Committee. For the reserpine study, male Sprague-Dawley rats (n=5-6 per group) weighing 200-250 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The rats were acclimated for one week prior to testing. The study protocol was approved by the Animal Care and Use Committee of Eli Lilly and Co.

Drugs. (-)-OSU6162 and ACR16 were synthesized at Eli Lilly, Indianapolis, IN, while haloperidol was obtained from Sabex Inc., Boucherville, QC, Canada. All drugs were dissolved in 1% glacial acetic acid and were administered subcutaneously (s.c.) in a volume of 1 ml/kg of body weight. [^{3}H] Raclopride (Perkin Elmer Life Sciences, Boston, MA) was used as the radioligand for the occupancy study and was administered intravenously (i.v.) via the tail vein. d-Amphetamine sulphate was obtained from US Pharmacopoeia and dissolved in physiological saline and administered subcutaneously in a volume of 1ml/kg for the locomotor experiments. Reserpine (RBI, Natick, MA) was dissolved in a few drops of glacial acetic acid and made up to volume in 5% glucose solution. The l-aromatic amino acid decarboxylase inhibitor NSD1015 HCl (3 – hydroxybenzylhydrazine, Sigma, St Louis) was dissolved in saline. Both reserpine and NSD1015 were injected in a volume of 5 ml/kg subcutaneously.

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Striatal Dopamine D_2 Receptor Occupancy Experiments. (-)-OSU6162 (3-120 mg/kg), ACR-16 (10-240 mg/kg) and haloperidol (0.025-1 mg/kg) were administered to rats to obtain a dose response of D_2 receptor occupancy levels, one hour after drug administration. Animals (n=5) were randomly assigned to each dose level. Thirty minutes prior to sacrifice all animals received an intravenous injection of [^{3}H] raclopride (7.5µCi/rat, in a volume of 0.4 ml of 0.9% w/v NaCl solution) to determine D_2 occupancy. Animals were sacrificed by decapitation and striata and cerebellum were rapidly dissected. Approximately one third of the cerebellum and the left and right striata pooled as a single sample were collected. The tissue was dissolved in 2 ml of Solvable® (Canberra Packard Canada, Montreal, QC, Canada) and was gently agitated for 24hours. Scintillation fluid was added and the vials were allowed to shake for another 24hours. Radioactivity was determined using liquid scintillation spectrometry using a Beckman Coulter LS5000 CE liquid scintillation counting system. To obtain an index of the binding potential of the dopamine D_2 receptors, the ratio of striatum minus cerebellum (index of specific binding)/cerebellum (index of free and non-specific binding), a clinical method validated for experimental animals was used (Wadenberg et al., 2000). To determine D_2 occupancies as a function of time (-)-OSU6162 (30 mg/kg), ACR-16 (60 mg/kg) and haloperidol (0.5 mg/kg) were evaluated at different time points. The occupancy induced by the drug of interest was calculated using the formula: $\%Occupancy = 100 \times (D_2BP_{controls} - D_2BP_{drug} / D_2BP_{controls})$; where $D_2BP_{controls}$ is the pooled D_2 binding potential of all the control animals and D_2BP_{drug} is the D_2 binding potential of a drug treated animal. Occupancy curves and the ED_{50} values (dose at which

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50% receptors are occupied) were determined using the non-linear regression equation representing a rectangular hyperbola [y=ax/(b+y)] using Sigma Plot®.

Catalepsy. Animals used for the occupancy experiments were also used to measure catalepsy. Catalepsy was measured 10 minutes before sacrifice. Animals were placed on an inclined grid (60º) and the time the animals remained immobile (excluding the first 30 seconds) was used as an index of catalepsy (on a scale 0 - 5 in which time was a square root transformation: 0 = 0–0.08, 1 = 0.09–0.35, 2 = 0.36–0.80, 3 = 0.81–1.42, 4 = 1.43–2.24, 5= >2.24 minutes (Wadenberg et al., 2000). An animal with a score of 2 or greater was considered cataleptic.

Behavioral stabilization - locomotor activity. Custom-made locomotor activity boxes similar to the home cages (clear Plexiglas® 27 x 48 x 20 cm), equipped with a row of six photocell beams placed 3 cm above the floor of the cage were used. A computer was used to record disruption of the photocell beams. Two sets of the experiment were carried out. In case of amphetamine-induced hyperactivity, 30 minutes after drug administration, rats were placed in the locomotor activity boxes in order to habituate for a period of 30 minutes. After 30 minutes, d-amphetamine (1.5 mg/kg, s.c.) was administered and locomotor activity was monitored for a period of 60 minutes. The ED_{50} value was the dose that was required to inhibit 50% of locomotor activity counts, recorded over the period of 60 minutes, with respect to vehicle treated amphetamine administered animals and was calculated using non-linear regression using Sigma Plot®. In the case of locomotor activity in habituated rats, they were habituated to the locomotor boxes for

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three consecutive days for a period of one hour each day. On the day of the experiment rats were allowed to habituate to the locomotor activity cage for 30 minutes followed by an injection of the drug. Locomotor activity was monitored for a period of one hour. Each dose of drug testing had a minimum of five animals. (-)-OSU6162 (3–60 mg/kg), ACR16 (3–60 mg/kg) and haloperidol (0.01-0.5 mg/kg) were tested in amphetamine induced hyperlocomotor activity, while (-)-OSU6162 (1–60 mg/kg), ACR16 (3–60 mg/kg) and haloperidol (0.01-0.1 mg/kg) were tested in habituated rats.

Striatal DOPA levels in reserpinized rats. Rats were first dosed with reserpine (5 mg/kg, s.c., 18-20 hours prior) to deplete their monoamine vesicular stores. Test compounds were given subcutaneously, 30 minutes before the L-aromatic amino acid decarboxylase inhibitor NSD 1015 (100 mg/kg, s.c.), and rats were sacrificed 30 minutes later. As a result of prolonged synaptic depletion of dopamine due to reserpinization, the synthesis-regulating dopamine D_2 autoreceptors develop a certain degree of supersensitivity and are hence sensitive to dopamine agonists/partial agonists (Svensson et al., 1991). In the presence of an L-aromatic aminoacid decarboxylase inhibitor, L-DOPA accumulates and the amount of L-DOPA accumulated during a 30 minute period is taken as a measure of the synthesis rate of dopamine. Partial and full agonists in reserpinized rats decrease this endpoint. Each treatment group had a minimum of five animals. Striatal levels of L-DOPA were measured using standard HPLC-ED techniques.

Conditioned Avoidance Response. Rats were trained and tested in a 2-way active avoidance apparatus (custom made shuttle boxes - Med Associates® St. Albans, VT).

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The boxes contained a sound and light attenuating shell in which two compartments of equal size were separated by a translucent partition with a single opening to ensure a two-way active avoidance setting. The shuttle boxes were enabled with a tilting grid floor and micro-switch detection. An 80-dB white-noise served as a conditioned stimulus, while a 0.6 mA foot shock served as the unconditioned stimulus. The rat's location was detected by activation of micro-switches fixed at the base of each compartment and programs running on a computer controlled the operations of the task. Animals that moved to the other side of the box within the period of the conditioned stimulus (10 seconds) were noted as having made an "avoidance" response. Those who escaped the shock in the next 20 seconds were termed as having "escaped", and those not escaping within the total 30 seconds were termed as "escape failures" (Wadenberg et al., 2001). Rats were trained for five days before drug testing. A performance criterion of greater than 80% avoidance after the five-day training served as the basis for selecting rats that were used for drug testing. The entire protocol as well as recording of the animal's performance was controlled by Med Associates® computer routines. (-)-OSU6162 (30, 60 & 120 mg/kg n = 5), ACR16 (30, 60 & 120 mg/kg n = 7) and haloperidol (0.01, 0.05 & 0.15 mg/kg n = 6) were administered in a manner such that animals in each drug group served as their own controls in a within-subject design. CAR was measured at 0, 20, 90, 240 minutes and finally at 24 hours after drug administration and an interval of 2 days served as a washout period.

Fos Immunohistochemistry. Rats were deeply anaesthetized with sodium pentobarbital (100 mg/kg i.p.) two hours after drug administration. They were perfused through the

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ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Whole brains were collected and post-fixed in 4% paraformaldehyde for 24 hours, transferred to sucrose solutions (10% for 2 hr, 20% for 12 hr and 30% for 24 hr) and then dried and stored at –80°C until processing. Free-floating, forty-micron cryostat sections were incubated with a polyclonal primary antiserum raised in rabbit against the Fos peptide (4-17 amino acids of human Fos; Oncogene Research Products, Cambridge, MA), diluted 1:5000 for 48 hours at 4°C. The tissue sections were then exposed to biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, California, USA), which was followed by incubation with horseradish peroxidase avidin-biotin complex (Vector Laboratories, Burlingame, CA) to visualize the Fos staining. Fos-immunoreactive nuclei were counted by an observer blind to treatment conditions, within a 400 x 400 μm grid at a magnification of 100x in the shell of the nucleus accumbens and dorsolateral striatum (bregma 1.70 to 1.00; Paxinos and Watson, 2nd ed, 1986) (Robertson et al., 1994) using an MCID M5 system (Imaging Research, St. Catherines, Ontario, Canada). Cell counts were obtained from at least 3 separate brain sections for each brain, obtained from four subjects per group. (-)OSU6162 (10, 30, 60, 120 mg/kg), ACR16 (10, 30, 60, 120 mg/kg) and haloperidol (0.01, 0.05, 0.5, 1 mg/kg) were tested for Fos protein expression.

Prolactin estimation. Prolactin levels were measured using plasma collected from rats sacrificed for the D_2 receptor occupancy/catalepsy experiments. Plasma samples were stored at -80ºC until they were assayed. Prolactin levels (ng/ml) were measured using a rat prolactin enzyme immunoassay kit (ALPCO Diagnostics®, Windham, NH).

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Results

Occupancy and catalepsy. All three drugs showed dose-dependent striatal D_2 receptor occupancy (Figure 1). (-)-OSU6162 over a range of 3-120 mg/kg (37-90% occupancy) showed a dose dependent occupancy of D_2 receptors with an $ED_{50\%}$ of 5.27 mg/kg (CI: 3.09–7.45) at the one-hour time point. ACR16 on the other hand showed a dose dependent occupancy of D_2 receptors over a range of 10-240 mg/kg, (35-90% occupancy) with an ED_{50} of 18.99 mg/kg (CI: 11.59-26.39), one hour after administration. Similarly haloperidol over a dose range of 0.025-1 mg/kg showed an occupancy range of 53 to 90% with an ED_{50} of 0.02 mg/kg (CI: 0.012–0.028). (-)-OSU6162 did not show catalepsy in the dose range used for the occupancy study, but in case of ACR16 weak catalepsy was observed in one out of five animals at a dose of 120 mg/kg. Haloperidol treated animals showed catalepsy when D_2 occupancy was ≥0.1 mg/kg (>80% D_2 receptor occupancy, Figure 1). The results of the time course experiment are compiled in Table 1; while haloperidol (0.5 mg/kg) occupied D_2 receptors for a very long time, (-)-OSU6162 (30 mg/kg) had the shortest D_2 occupancy time course.

Behavioral Stabilization – Locomotor activity. All three drugs were effective in decreasing amphetamine-induced hyperlocomotion (Figure 2). The ED_{50} (95% CI) values for (-)-OSU6162, ACR16 and haloperidol were 44.7 (39–52), 28.2 (20–34) and 0.05 (0.04-0.06) mg/kg, respectively. In the 30-minute habituation phase of locomotor activity

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before administration of amphetamine, haloperidol at doses of 0.1 and 0.5 mg/kg significantly ($p<0.05$) reduced exploratory locomotor activity by 45 and 67%, respectively from vehicle controls, ACR16 did so only at the highest tested dose (60mg/kg 42% from vehicle controls), while (-)-OSU6162 upto a dose of 60mg/kg did not significantly decrease exploratory locomotor activity compared to pooled vehicle controls in the tested doses indicating subtle effects of dopamine stabilizers on this behavior (One way ANOVA $F(11,74) = 5.48$ followed by two-sided Dunnett post hoc test). (-)-OSU6162 and ACR16 were able to significantly increase locomotor counts in rats habituated to the activity cages in similar dose ranges required to decrease amphetamine-induced hyperlocomotion and occupy striatal D_2 receptors (Figure 2). (-)-OSU6162, 60 mg/kg increased locomotor activity by 112% from baseline in habituated rats and at the same dose decreased amphetamine-induced locomotor activity by 65%. ACR16 on the other hand at a dose of 30 mg/kg increased locomotor activity in habituated rats by 87% while decreasing locomotor activity by 64% in animals stimulated by amphetamine. So over an occupancy range of 37–87% (3–60 mg/kg) for (-)-OSU6162 and 35–74% (10–60 mg/kg) for ACR16, we observed both inhibitory and stimulatory effects on locomotion depending upon the prevailing baseline motor activity. However, in contrast to the dopamine stabilizers, haloperidol (over an occupancy range of 33–78%, 0.01–0.1 mg/kg) potently inhibited psychostimulant activity and induced catalepsy, but failed to activate habituated animals showing low baseline activity.

Striatal DOPA levels in reserpinized rats. The dopamine agonist apomorphine (0.5 mg/kg) produced an 80% suppression of striatal DOPA accumulation, while both (-)-

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OSU6162 (30 mg/kg) and ACR16 (30, 60mg/kg) showed a weak, but statistically significant increase of DOPA levels above vehicle treated controls (Figure 3).

Conditioned Avoidance Response. Rats were administered (-)-OSU6162 (30 mg/kg, 60 mg/kg; 100 mg/kg) using a within-subject design. The mean values of inhibition of avoidance based on repeated observations (20, 90, and 240 min after drug injection) of the same five animals are shown in Figure 4. No escape failures were observed in the three tested doses of (-)-OSU6162. The maximal inhibition of avoidance (49%) was observed 20 min post drug administration for a dose of 100 mg/kg and hence the ED_{50} was not determined at any time point. In the case of ACR16, rats were administered doses of 30, 60 and 120 mg/kg and the values for inhibition of avoidance are shown based on repeated observations (20, 90, 240 min and 24hr after ACR16 injection) of 7 animals (Figure 4). No escape failures were observed at the dose of 30 mg/kg. At 60 mg/kg escape failures were observed (1/7 at 20 min; 5/7 at 90 min; 1/7 at 240 min and all the rats recovered at 24hr test point). In the case of 120 mg/kg escape failures were observed as follows: 2/7 at 20min; 6/7 at 90 min; 6/7 at 240 min and all the rats recovered at 24hr test point. The ED_{50} value at 90 min was determined to be 39.83 (CI 95%: 24.5–52.8) mg/kg using GraphPad® software which was equivalent to ~ 65% D_2 receptor occupancy. Haloperidol administered at doses of 0.01, 0.05 and 0.15 mg/kg inhibited CAR and did not induce escape failures at the time points tested (20, 90, 240 min and 24hr after drug injection) (Figure 4). Its ED_{50} at 90 minutes was 0.019 (CI 95%: 0.002–

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0.03) mg/kg equivalent to ~50% D_2 receptor occupancy determined using GraphPad® software.

Fos Immunohistochemistry. All three drugs significantly induced Fos in the nucleus accumbens as well as dorsolateral striatum (Figure 5). The amount of Fos induced by 30 mg/kg of (-)-OSU6162 & 60 mg of ACR16 was equivalent to 0.1 mg/kg of haloperidol in the nucleus accumbens. However, inspite of significant Fos induction in the nucleus accumbens with (-)-OSU6162, inhibition of CAR was minimal. Fos induction in the dorsolateral striatum of animals treated with (-)-OSU6162 as well as ACR16 did not translate into induction of catalepsy, as was the case for haloperidol. Comparing Fos induction to occupancy percentages, both (-)-OSU6162 and ACR16 significantly induced Fos in the nucleus accumbens as well as in the dorsolateral striatum at doses that exceeded 60% D_2 receptor occupancy.

Plasma prolactin measurements. (-)-OSU6162 and haloperidol showed dose-related prolactin induction (Figure 6). In the haloperidol group, one rat in the 0.05 mg/kg treatment group was a significant outlier (prolactin value of 140 ng/ml; Grubbs test Z = 1.71, $p<0.05$) and was excluded from the calculations. ACR16 elevated prolactin values minimally and a statistically significant increase was noted only at the dose of 120 mg/kg (Figure 4). In the case of (-)-OSU6162 the dose of 10 mg/kg correlated to a central D_2 receptor occupancy of 64% and induced significant prolactin levels, while in the case of ACR16 the dose of 60mg/kg which induces a D_2 receptor occupancy of 75% did not significantly increase plasma prolactin levels.

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Discussion

The dopamine stabilizers (-)-OSU6162 and ACR16 show a tone-dependent mixture of stimulatory and depressant effects in locomotor activity models, lending credence to the view that their 'dopamine stabilization' properties can be identified and replicated (Figure 2). Despite their reported low in vitro D_2 receptor affinities the drugs show remarkable D_2 occupancies in vivo in a dose-dependent manner, reaching saturation, consistent with an orderly binding to the dopamine D_2 receptors (Figure 1). As with other antipsychotics, the drugs did show activity in the CAR model, blocked d-amphetamine-induced hyperactivity and induced Fos in limbic regions. However, the effects of these drugs were clearly different from the standard D_2 blocker haloperidol, as catalepsy was not observed in any animal receiving (-)-OSU6162 and only one animal treated with 120 mg/kg of ACR16 for which D_2 receptor occupancies in vivo exceeded 80%. At this degree of striatal D_2 receptor occupancy both typical and atypical antipsychotics act as full antagonists inducing catalepsy in rodents (Wadenberg et al., 2001; Kapur et al., 2003). Also these behavioral stabilizers had minimal inhibitory effects on the exploratory phase during locomotor experiments in rats that were not habituated to locomotor boxes. Our findings of significant D_2 receptor occupancy with (-)-OSU6162 in vivo are in line with previous findings showing that this compound displaced the doapamine agonist Di-PR-5,6-ADTN in vivo in the rat striatum after subcutaneous dosing (Sonesson et al., 1994) and showed full occupancy of striatal D_2 receptors labeled with raclopride after intravenous dosing to anesthetized monkeys (Ekesbo et al., 1999). The implication of

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the current findings regarding the potential of these drugs to act as antipsychotics in the clinic as well as limitations and caveats are discussed below.

The high level of in vivo receptor occupancy with relatively modest effects on motor activity made us consider whether these compounds may have intrinsic efficacy (i.e. whether they are partial dopamine agonists). Partial D_2 agonists (e.g. aripiprazole or preclamol) have little or no effect on DA turnover, while full D_2 antagonists cause a significant increase in DA turnover (Semba et al., 1995; Oshiro et al., 1998; Nakai et al., 2003; Jordan et al., 2004). To rule out partial agonism by (-)-OSU6162 and ACR16, we used a dopamine-depleted (reserpinized) animal preparation which is a particularly sensitive assay for in vivo D_2 agonist effects (Yasuda et al., 1988; Petterson et al., 2002). We observed that apomorphine, a full D_2 receptor agonist produced nearly 80% suppression of striatal DOPA accumulation in reserpinized rats (Figure 3). While Sonesson et al (1994) reported no effects of (-)-OSU6162 (30 mg/kg) on DOPA accumulation in the striatum of reserpinized rats we found that both (-)-OSU6162 and ACR16 showed weak but statistically significant increases (10-40%) in DOPA accumulation. The ability of (-)-OSU6162/ACR16 to slightly enhance dopamine synthesis rate above reserpine control levels may indicate the presence of synaptic dopamine. Nevertheless, the present data confirm that the two compounds lack intrinsic activity in dopamine autoreceptors displaying a certain degree of supersensitivity (Hjorth et al., 1988). Dopamine antagonists such as haloperidol are reported not to affect dopamine synthesis rate in reserpinized rats (Johansson et al., 1985). In line with the lack of direct D_2 receptor agonist effects of ACR16 and (-)-OSU6162, these compounds

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are reported to elevate both synthesis, release and metabolism of dopamine in normal rats, as do other full D_2 receptor antagonists (Sonesson et al., 1994; Petterson et al., 2002). In contrast to dopamine agonists (full or partial), (-)-OSU6162 failed to induce behavioral activation in reserpinized rats (Sonesson et al., 1994) and to induce rotational behavior in 6-hydroxy dopamine lesioned rats (Nichols et al., 2002), while it blocked rotational behavior induced by dopamine agonists such as quinpirole, apomorphine or L-DOPA in lesioned rats or monkeys without inducing strong akinesia or dystonia (Ekesbo et al., 1997; Ekesbo et al., 2000; Nichols et al., 2002). Finally, we compared (-)-OSU6162 and ACR16 with haloperidol regarding their effects on plasma prolactin levels. Like haloperidol, (-)-OSU6162 and ACR16 induced dose-dependent increase (>100% over baseline) in prolactin, which further confirms their lack of direct agonist activity at dopamine D_2 receptors (Figure 6). In summary, our data strongly suggest that both compounds act as full dopamine D_2 receptor antagonists in vivo without evidence for intrinsic agonist activity. However, we cannot rule out the possibility that the difference in behavioral stabilizing property between these drugs and haloperidol could be due to actions at receptors other than D_2, although significant in vivo or in vitro affinities for other neurotransmitter receptors have not been found (Sonesson et al., 1994; Petterson et al., 2002).

Both compounds showed clear evidence of antipsychotic-like efficacy in the animal models used. In the CAR assay, which is believed to predict clinical efficacy against positive symptoms (Janssen and Awouters, 1994), ACR16 was clearly more efficacious than (-)-OSU6162 (Figure 5). This difference could be explained by a comparable D_2

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receptor occupancy of (-)-OSU6162 and ACR16 inspite of higher in-vitro affinity exhibited by (-)-OSU6162. Also (-)-OSU6162 has a shorter time course of D_2 receptor occupancy and that might explain its low efficacy (Table 1). Elevated prolactin levels due to (-)-OSU6162, compared to ACR16 (Figure 6) at comparative D_2 occupancy levels, could mean differences in its kinetics of distribution (Kapur et al., 2002). Our D_2 receptor occupancy data for ACR16 is similar to those recently published by Carlsson and Carlsson (2006) in which about 60-70% displacement of raclopride at doses of 150 μmol/kg (approximately 50 mg/kg) was observed. It should be pointed out that the doses at which the compounds are active in the CAR assay are comparatively high (60-120 mg/kg) when compared to clinically used agents (Wadenberg et al., 2001; Kapur et al., 2003). However, initial pilot studies with (-)-OSU6162 (100 mg daily dose) suggest antipsychotic activity in schizophrenic patients when administered as an add-on to standard treatments (Gefvert et al., 2000; Carlsson et al., 2004). It will certainly be of interest to see whether compounds of this class show antipsychotic activity when given as stand-alone therapies. Additional evidence of antipsychotic-like activity of ACR16 and (-)-OSU6162 include preferential Fos induction in the nucleus accumbens compared to the dorsolateral striatum indicating a certain limbic selectivity (Figure 5). The high Fos induction due to (-)-OSU6162 compared to ACR16 (in a similar D_2 occupancy range) remains puzzling and could be due to action at targets unaccounted for in the present study. Also, both compounds completely reversed d-amphetamine induced hyperactivity without inducing strong hypoactivity or catalepsy. ACR16 and in particular (-)-OSU6162 showed evidence of enhanced plasma prolactin levels one-hour post treatment. Data from time course studies is needed to reveal whether these effects are sustained and therefore

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of potential clinical significance. Recently (-)-OSU6162 has also been shown to reduce apomorphine and amphetamine induced behavior in subhuman primates that further substantiates a role for low affinity dopamine D_2 antagonists in the treatment of pscyhosis (Brandt-Christensen et al., 2006).

It remains a puzzle as how a D_2 blocker could lead to both an increase as well as a decrease in locomotor activity when haloperidol does not do it. The only other antipsychotic tested in a similar fashion that showed a similar profile was remoxipride (Sonesson et al., 1994), which is also a low affinity D_2 antagonist. It is not clear if low affinity to D_2 receptor is solely responsible for such actions, but clearly, these compounds showed weak but significant behavioral activating properties in habituated rats both in our hands as well as in other laboratories (Sonesson et al., 1994; Waters et al., 2002). Recently, a mechanism by which these compounds exert their stabilizing properties based on differences in extrasynaptic versus synaptic dopamine neurotransmission has been proposed (Carlsson and Carlsson, 2006). Also a recent study of these compounds showed reversal of (+)-MK-801 induced behavioral impoverishment in mice and social withdrawal in rats (Nilsson et al., 2004; Rung et al., 2005). This suggests that the compounds may possess activity against negative symptoms including social withdrawal. Anecdotal clinical data generated with (-)-OSU6162 lends support to these findings (Gefvert et al., 2000; Carlsson et al., 2004). As is the case for atypical antipsychotics (-)-OSU6162 (Sonesson et al., 1997; Waters et al., 2002) were reported to enhance dopamine release in the prefrontal cortex of the rat in vivo, a key factor in improving cognitive deficits seen in schizophrenic patients (Abi-Dargham and Moore, 2003). Taken together,

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these findings indicate that the dopamine D_2 receptor stabilizers could be effective in treating positive as well as negative and cognitive symptoms in schizophrenia.

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Legends for Figures

Figure 1

Striatal D_2 receptor occupancy one hour after administration of (-)-OSU6162, ACR16 or haloperidol (n = 5 for each dose level). The curves were generated using a non-linear regression equation representing a rectangular hyperbola [y=ax/(b+x)] using Sigma Plot® software.

Figure 2

The upper panel shows effects of (-)-OSU6162 (n=5 for each dose level), ACR16 (n = 5 for each dose) and haloperidol (n = 6 for each dose) (mg/kg) on locomotor activity measured for one-hour duration after amphetamine or saline administration and expressed as Mean ± SEM. The drugs or vehicle were administered 30 minutes prior to amphetamine or saline challenge. **$p<0.001$, One-way ANOVA $F(11,78) = 10.63$; post hoc Dunnett (two sided) with respect to pooled amphetamine treatment. The lower panel shows effects of (-)-OSU6162 (n = 5 for each dose level), ACR16 (n = 6 for each dose level) and haloperidol (n = 6 for each dose level) on rats habituated to locomotor activity cage environment. Locomotor activity was measured for one hour after drug injection and counts are expressed as Mean ± SEM **$p<0.001$, *$p<0.05$ One way ANOVA $F(14,90) = 29.97$; post hoc Dunnett (two sided) with respect to pooled vehicle treatment.

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Figure 3

Effect of apomorphine, (-)-OSU6162 and ACR16 (n = 5 for each dose for all the drugs) on the dopamine synthesis rate in reserpinized animals in the rat striatum. Values are expressed as % of vehicle treated controls, Mean ± SEM *$p<0.01$ One way ANOVA $F(5,29) = 52.81$; post hoc Dunnett (two sided) with respect to vehicle treatment.

Figure 4

Effect of (-)-OSU6162 (n = 5), ACR16 (n = 7) and haloperidol (n = 6) on the performance of conditioned avoidance response in rats after single subcutaneous injection. The animals served as their own controls using a within-subject design. Values of percentage inhibition of avoidance are expressed as Mean ± SEM. The avoidance values were analyzed for each drug in a repeated measures analysis of variance with dose (vehicle, three drug doses) as a within subject factor for each time point separately. If the sphericity assumption was not met Huynh-Feldt correction was applied and the main effect of dose was significant at least at one time point for all the drugs. Post hoc comparisons were performed using the Bonferroni adjustment for multiple comparisons and the level of significance indicated in the figure is that with respect to vehicle treatment (*$p<0.05$).

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Figure 5

Fos expression in the nucleus accumbens and dorsolateral striatum due to drug treatment (n = 4 for each dose). Rats were killed 2h after drug administration and Fos immunoreactive nuclei expressed as Mean ± SEM was counted within a 400 x 400 µm grid in the specified brain regions * $p < 0.005$ One way ANOVA $F(13,50) = 41.69$; post hoc Dunnett (two-sided) with respect to pooled vehicle control of nucleus accumbens. $^{\#}p < 0.05$ One way ANOVA $F(13,50) = 19.43$; post hoc Dunnett (two-sided) with respect to pooled vehicle treatment of dorsolateral striatum.

Figure 6

Plasma prolactin levels after (-)-OSU6162, ACR16 or haloperidol treatment measured from plasma samples obtained from the occupancy experiment one-hour after subcutaneous administration (minimum of n = 4 for each dose). Values are expressed as Mean ± SEM $^{**}p<0.001$, $^{*}p<0.01$ one way ANOVA $F(9,58) = 15.04$; post hoc Dunnett (2-sided) with respect to the pooled vehicle control.

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Table

Table 1 Time course of striatal D_2 receptor occupancy of (-)-OSU6162, ACR16 and haloperidol (% occupancy)

Drug	1hr	2hr	4hr	6hr	8hr	24hr
(-)-OSU6162 30 mg/kg	84.23 ± 4.84	70.17 ± 3.54	29 ± 12.25	< 15%	n.d.	n.d.
ACR16 60 mg/kg	77.9 ± 2.93	72.49 ± 8.8	78.4 ± 19.01	47.58 ± 4.07	n.d.	n.d.
Haloperidol 0.5 mg/kg	94.29 ± 1.45	90.46 ± 4.45	87.92 ± 2.17	n.d.	79.73 ± 2.07	36.93 ± 13.28

Values are Mean ± S.D. of striatal D_2 receptor occupancy values obtained using at least four rats at each dose level.

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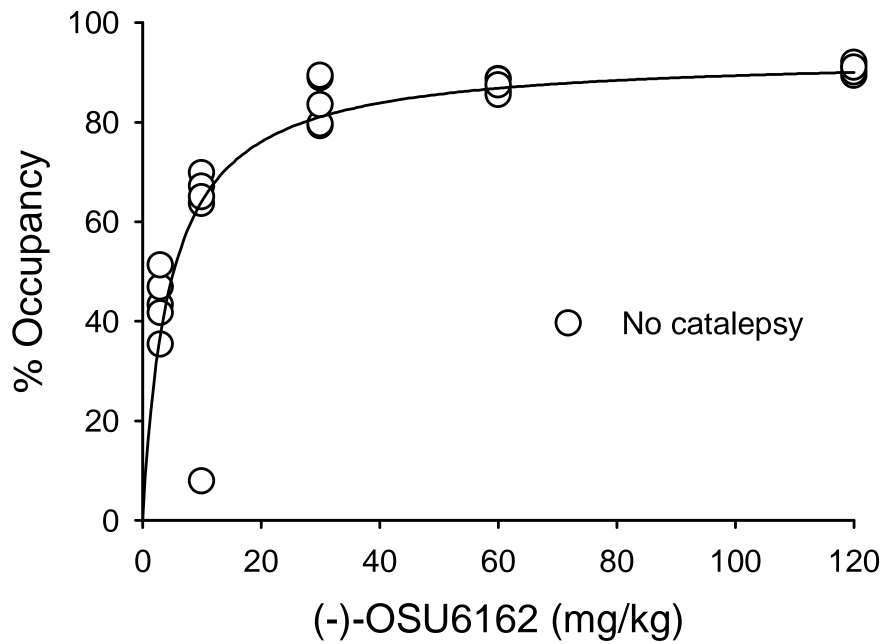
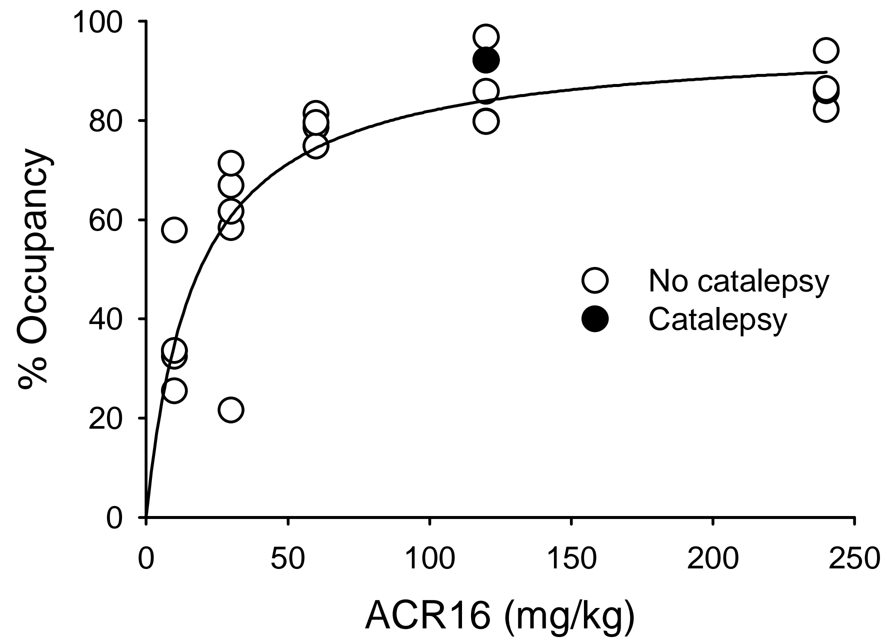
Figure 1





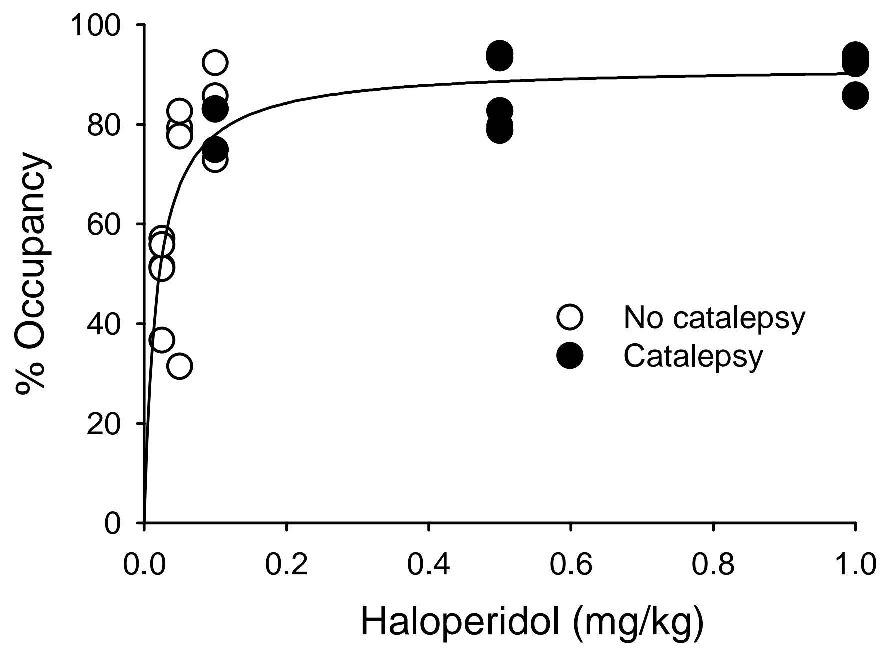


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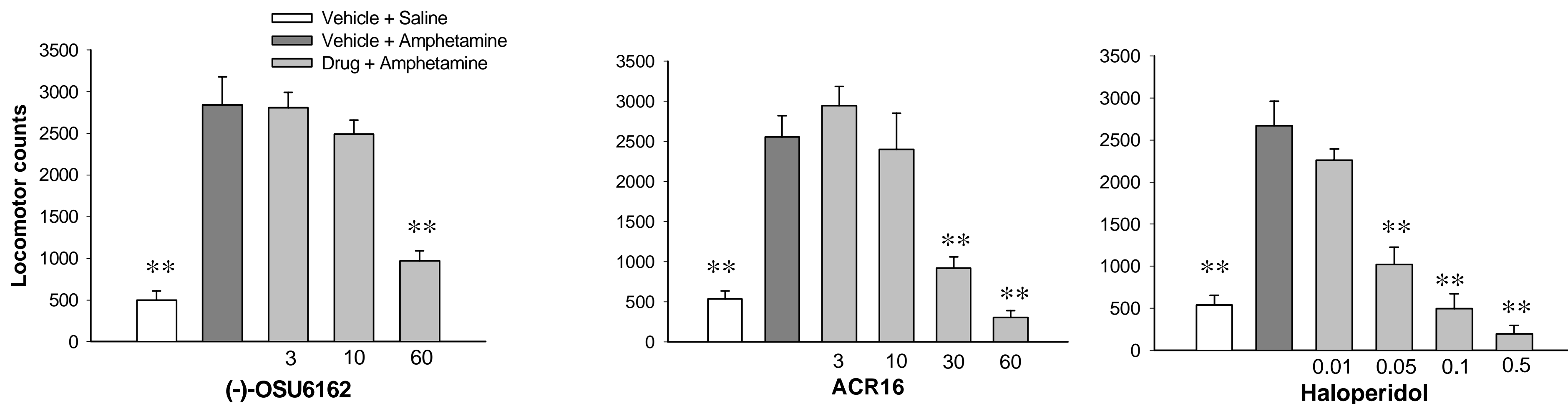
Figure 2



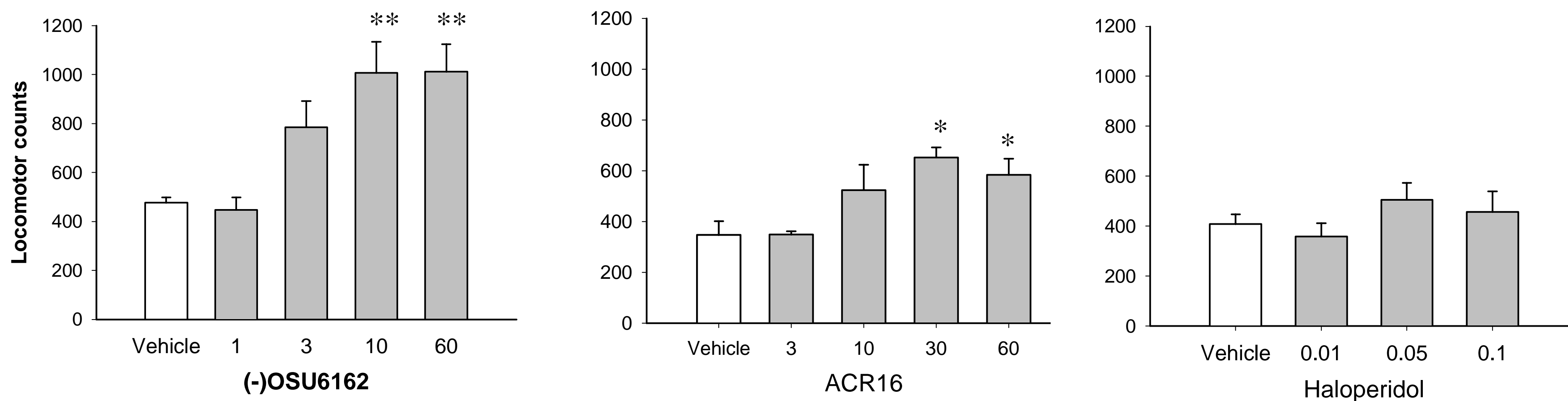


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Figure 3

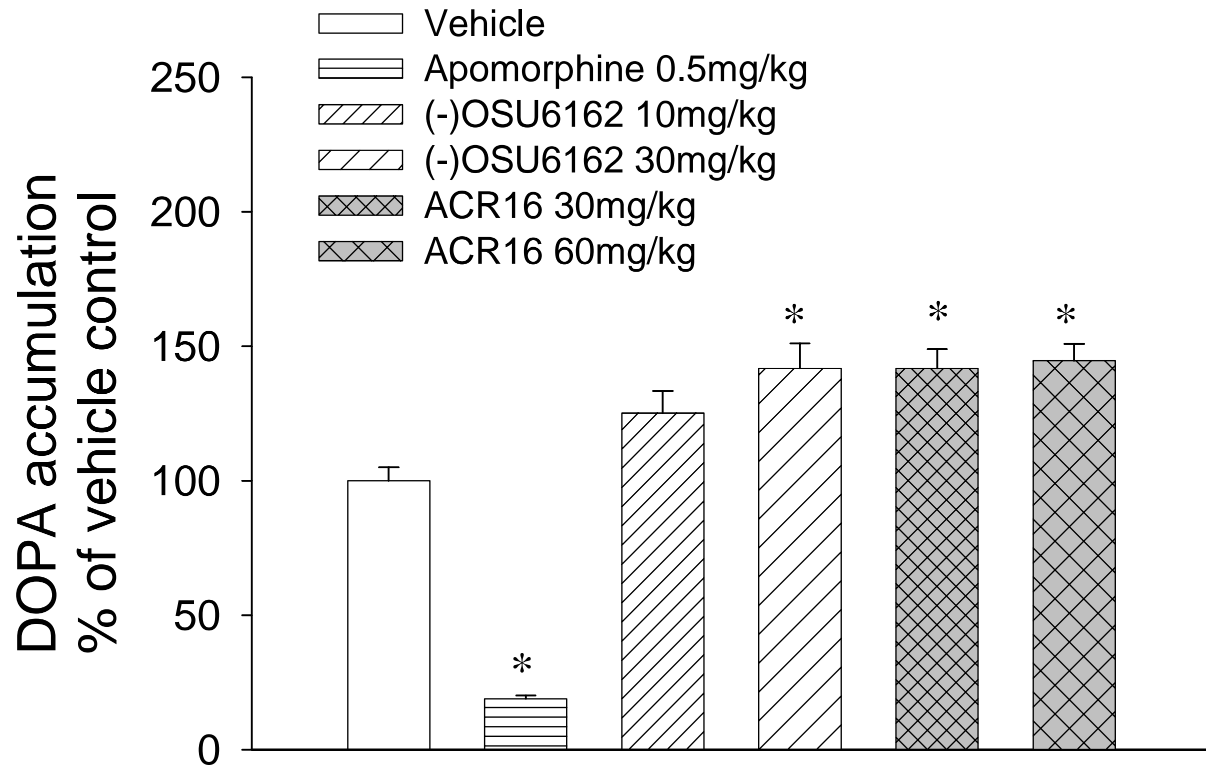

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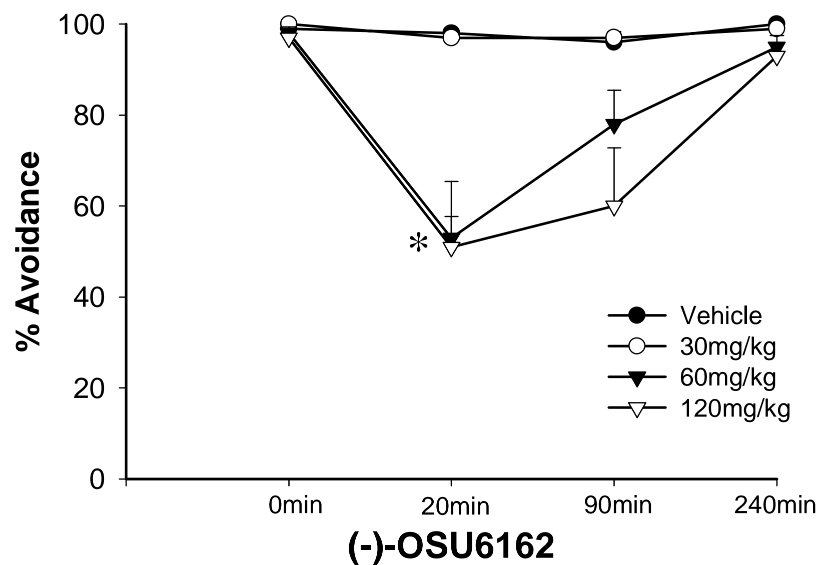
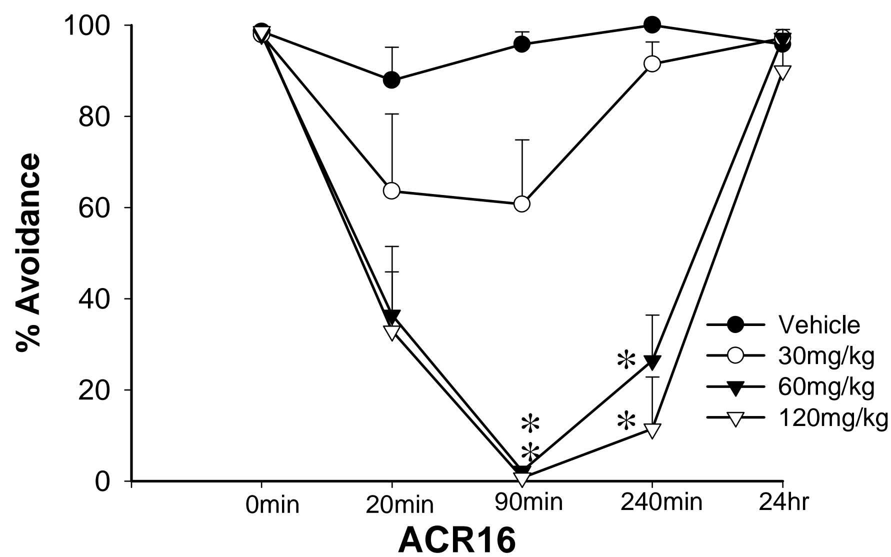
Figure 4





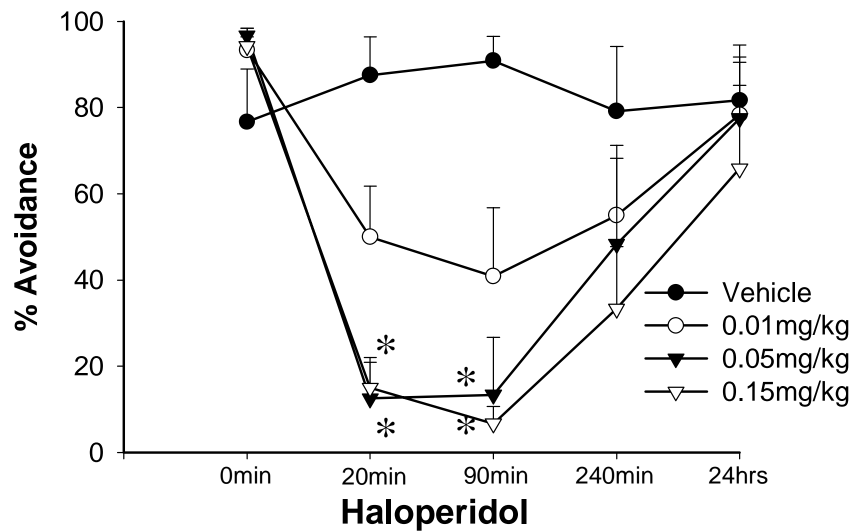


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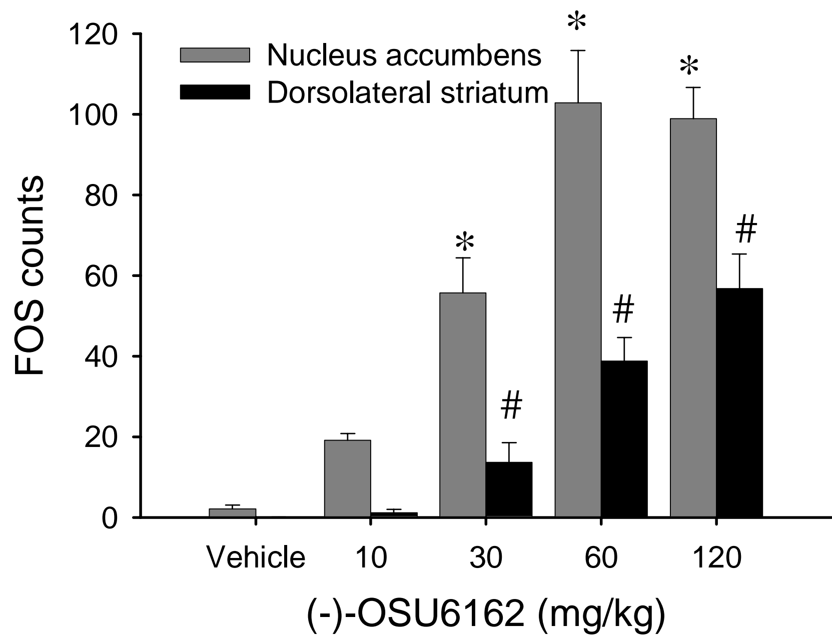
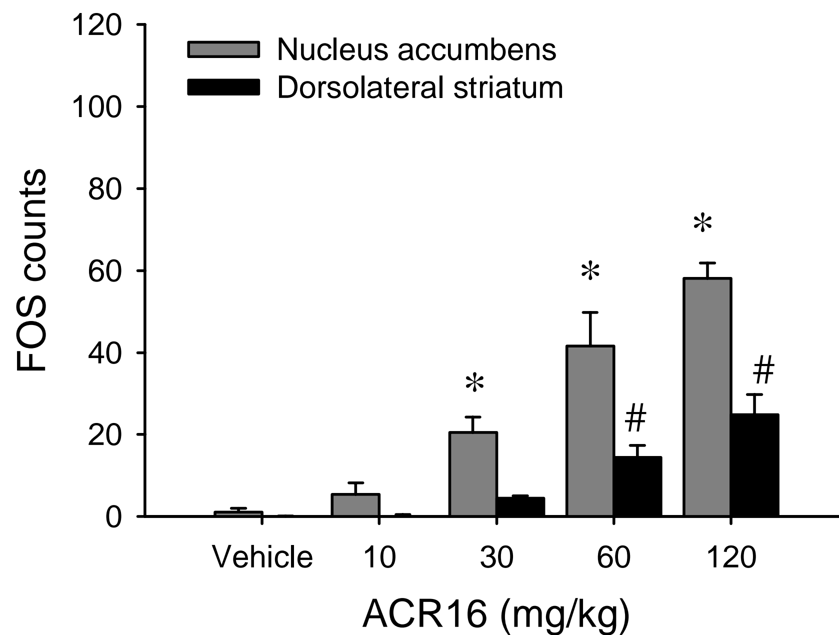
Figure 5





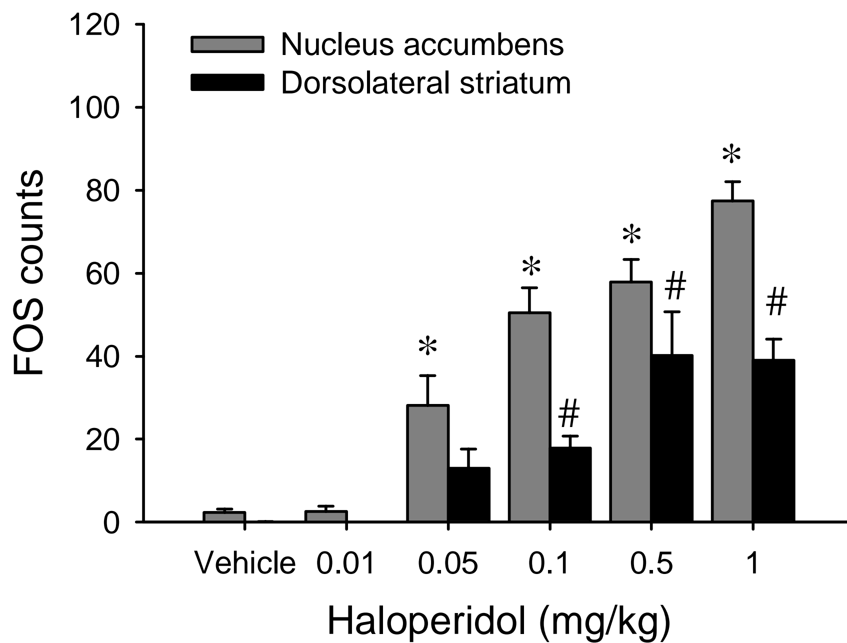


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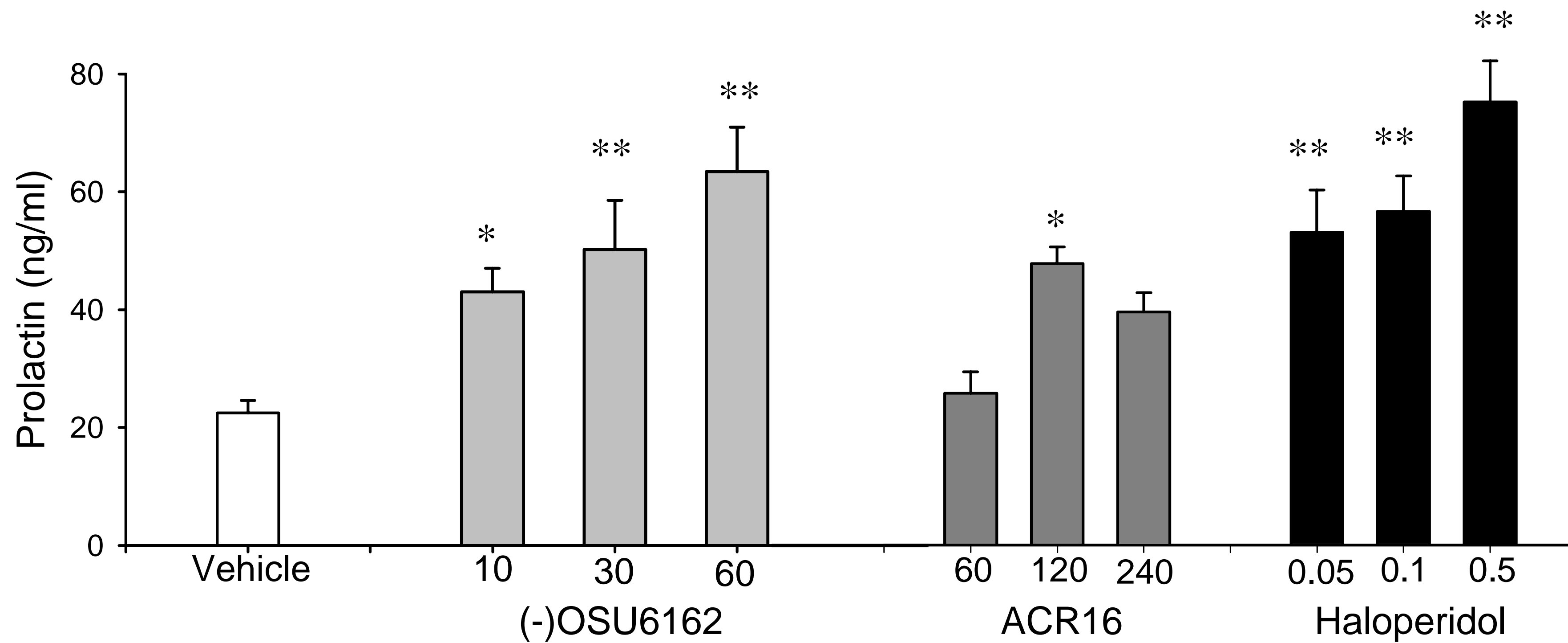
Figure 6



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