

Sex differences and serotonergic mechanisms in the behavioural effects of psilocin

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Psilocybin has recently attracted a great deal of attention as a clinical research and therapeutic tool. The aim of this paper is to bridge two major knowledge gaps regarding its behavioural pharmacology – sex differences and the underlying receptor mechanisms. We used psilocin (0.25, 1 and 4 mg/kg), an active metabolite of psilocybin, in two behavioural paradigms – the open-field test and prepulse inhibition (PPI) of the acoustic startle reaction. Sex differences were evaluated with respect to the phase of the female cycle. The contribution of serotonin receptors in the behavioural action was tested in male rats with selective serotonin receptor antagonists: 5-HT_{1A} receptor antagonist (WAY100635 1 mg/kg), 5-HT_{2A} receptor antagonist (MDL100907 0.5 mg/kg), 5-HT_{2B} receptor antagonist (SB215505 1 mg/kg) and 5-HT_{2C} receptor antagonist (SB242084 1 mg/kg). Psilocin induced dose-dependent inhibition of locomotion and suppression of normal behaviour in rats (behavioural serotonin syndrome, impaired PPI). The effects were more pronounced in male rats than in females. The inhibition of locomotion was

normalized by 5-HT_{1A} and 5-HT_{2B/C} antagonists; however, PPI was not affected significantly by these antagonists. Our findings highlight an important issue of sex-specific reactions to psilocin and that apart from 5-HT_{2A}-mediated effects 5-HT_{1A} and 5-HT_{2C/B} receptors also play an important role. These findings have implications for recent clinical trials. *Behavioural Pharmacology* 27:309–320

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Introduction

Psilocin (4-hydroxy-*N,N*-dimethyltryptamine) is an active metabolite of the naturally occurring tryptamine hallucinogen psilocybin (*O*-fosforyl-4-hydroxy-*N,N*-dimethyltryptamine) (Fantegrossi *et al.*, 2008), with the highest affinity to 5-HT₂ and 5-HT₁ serotonin receptors [see Tylš *et al.* (2014) for review]. It is typically found in a variety of hallucinogenic mushrooms and has a long history of ritual use (Hofmann, 2005; Carod-Artal, 2015), recently gaining a great deal of attention as a clinical research tool (pharmacological model of psychosis) and a potential therapeutic agent for depression in terminal patients, obsessive-compulsive disorder, addictions and cluster headaches (Tylš *et al.*, 2014). This provides a clear rationale for the investigation of less known/understood effects of psilocybin.

The acute effects of psilocybin in humans include profound changes in perception (dream-like states, illusions, hallucinations, synesthesia), altered self-perception, derealization and depersonalization, thought content disorder (magical thinking, unusual ideas or delusions) and sometimes also anxiety (Halberstadt and Geyer, 2013; Tylš *et al.*, 2014). Even though preclinical experiments cannot detect the effects mentioned above, they allow us to study many other domains (e.g. contribution

of receptor subtypes) in more detail. The most typical behavioural test used is the analysis of spontaneous behaviour in the open field, which enables the detection of changes in arousal, exploration, anxiety, habituation, etc. Alteration of these parameters could be, for example, a result of disrupted navigation as a consequence of perceptual changes, whereas avoidance of the open parts of the arena could reflect increased anxiety. Similarly, the measurement of a prepulse inhibition (PPI) deficit, a measure of sensorimotor processing, has been previously identified as an endophenotype of the effects of hallucinogens in animals.

Psilocin and psilocybin, as well as other serotonergic hallucinogens, induce comparable changes in various behavioural parameters in rodents. For example, they inhibit locomotor activity, induce signs of ataxia, reduce exploration, reduce time spent in the centre of the arena and suppress normal habituation to an environment (Delay *et al.*, 1959; Collins *et al.*, 1966; Schneider, 1968; Geyer *et al.*, 1979; Halberstadt *et al.*, 2011). Furthermore, typical signs of behavioural serotonin syndrome can be observed – for example, head twitch behaviour/wet dog shakes, flat body posture and backward walking (Ortmann, 1984; Fantegrossi *et al.*, 2008; Tylš *et al.*, 2014).

However, compared with other hallucinogens, they have inconsistent effects on sensorimotor processing: psilocybin increased the startle response in rats up to doses of 2 mg/kg, but decreased it at doses higher than 4 mg/kg (Davis and Walters, 1977; Geyer *et al.*, 2001; Halberstadt and Geyer, 2011). Although other hallucinogens mostly decreased PPI (Sipes and Geyer, 1995b; Ouagazzal *et al.*, 2001; Krebs-Thomson *et al.*, 2006; Palenicek *et al.*, 2008), an opposite effect was described for psilocybin up to doses of 4.5 mg/kg (Halberstadt and Geyer, 2011).

Most studies on hallucinogens show that the psychedelic activity, including psilocin/psilocybin, is mediated by stimulation of the 5-HT_{2A} receptor (Nichols, 2004; Tyls *et al.*, 2014). Head twitch behaviour, disruption of sensorimotor processing or discrimination studies with hallucinogens most typically relate to this receptor subtype (Fantegrossi *et al.*, 2008; Geyer and Vollenweider, 2008; Halberstadt *et al.*, 2011). However, psilocin binds to several other serotonergic and nonserotonergic receptors, with 5-HT_{1A} receptors being the most discussed as having important behavioural effects (Aghajanian and Hailgler, 1975; Winter *et al.*, 2007; Fantegrossi *et al.*, 2008; Halberstadt and Geyer, 2011; Tyls *et al.*, 2014).

Despite the fact that behavioural patterns and mechanisms of action of psilocin and psilocybin have been widely described, some topics have still been under-investigated, with sex differences in the behavioural response to the drug being among the most important. It is well known that women have a different sensitivity to the effects of centrally acting drugs (Marazziti *et al.*, 2013). Their emotionality fluctuates during their cycle, which is believed to be significantly mediated by the effects of steroids on the serotonergic system (Bancroft, 1995; Landen and Eriksson, 2003). It is therefore likely that psilocybin will have different effects in various parts of the female cycle. Even though no sex-related differences have been reported to date in human psilocybin studies (Studerus *et al.*, 2012), attenuation of some LSD (*N,N*-dimethyllysergamide) effects by female sex steroids has been described in humans (Krus *et al.*, 1961, 1967). Furthermore, the hypothesis that the response to psilocin would differ between sexes is supported by behavioural experiments with other mind-altering serotonergics, such as LSD and 3,4-methylenedioxymethamphetamine in rats (Meehan and Schechter, 1998; Palenicek *et al.*, 2010; Wallinga *et al.*, 2011; Kolyaduke and Hughes, 2013). These observations could be explained by sex steroid-mediated/dependent differences in the serotonin system (Cyr *et al.*, 2000; Birzniece *et al.*, 2002; Zhou *et al.*, 2002; Cosgrove *et al.*, 2007; Sumner *et al.*, 2007).

The second unanswered and underinvestigated question is the role of serotonin 5-HT_{2B/C} receptors in the mechanism of action of psilocin. Recent evidence shows that these receptors are also involved in the control of mood and anxiety (Kennett *et al.*, 1996; Duxon *et al.*,

1997; Martin *et al.*, 2014). According to ligand-binding studies, psilocin has a higher affinity to these receptors than to 5-HT_{2A} receptors (Blair *et al.*, 2000; Ray, 2010; Halberstadt and Geyer, 2011). Clarification of the role of these receptors would therefore contribute to the understanding of the potential antidepressant effect of psilocybin.

A better understanding in both of these areas is crucially important, because psilocybin is nowadays widely used in clinical trials [reviewed in Tyls *et al.* (2014)]. Therefore, to better understand the behavioural pharmacology of psilocin we designed our experiments specifically to identify the effect of sex and the female cycle and of 5-HT_{2B/C} receptors on behavioural changes induced by psilocin. Initially the effect of sex and different times of the female cycle on behavioural patterns in the open-field test and in the prepulse inhibition test was studied. Subsequently, in male rats only, the contribution of serotonin 5-HT_{1A} and 5-HT_{2A/B/C} receptors on locomotor activity and PPI was compared using selective antagonists.

Methods

Subjects

The experiments were performed on adult Wistar rats (SPF animals Hannover breed; MediTox, Konárovice, Czech Republic) weighing 200–250 g (males) and 150–180 g (females). The animals were acclimatized to local laboratory conditions for at least 7 days before the experiments. Rats had free access to a standard diet and water. They were housed in a 12 h light/dark regime at a temperature of 22°C–24°C. All of the testing was performed during the light phase. There were 8–12 animals in each experimental group. Each subject was tested only once. All the experiments respected the Guidelines of the European Union (86/609/EU) and followed the instructions of the National Committee for the Care and Use of Laboratory Animals.

Experimental schedule

Behavioural tests for locomotor activity, qualitative assessment of behaviour and sensorimotor gating were initially performed in both sexes and in relation to the oestrus phase of female rats. In the experiments designed to describe serotonergic mechanisms, locomotor activity and sensorimotor gating were evaluated in male rats only. Male rats from the above-mentioned experiments were also used for comparisons in this set of experiments for two reasons: (a) to minimize the total number of animals used (b) as all experiments were performed in parallel.

Identification of the oestrus phase in the female rats

To estimate the phase of the cycle on the day of testing, preliminary vaginal smears were taken at least 1 day earlier. On the day of testing, vaginal smears were taken from the female rats to verify the phase immediately after

the testing session. Each oestrus phase was determined microscopically according to the protocol followed by Marcondes *et al.* (2002), as in our previous studies (Bubenikova *et al.*, 2005; Palenicek *et al.*, 2010). A pro-oestrus smear consists of a predominance of nucleated epithelial cells, an oestrus smear primarily consists of anucleated cornified cells, a metoestrus smear consists of the same proportion of leucocytes, cornified and nucleated epithelial cells, and a dioestrus smear consists of a predominance of leucocytes. Female rats were then separated into two subgroups: pro-oestrus + oestrus (PE) and metoestrus + dioestrus (MD) females (Bubenikova *et al.*, 2005; Palenicek *et al.*, 2010).

Locomotor activity

Locomotor activity (trajectory length) was registered and analysed by an automatic video tracking system for recording behavioural activities (EthoVision Color Pro v. 3.1.1; Noldus, Wageningen, the Netherlands). A square black plastic box arena (68 × 68 × 30 cm), which was a novel environment for all animals, was placed in a soundproof and uniformly lit room. Locomotor activity was registered after administration of psilocin alone or with 5-HT antagonists or their respective vehicles for 30 min. The total distance moved, time spent in the centre of the arena and thigmotaxis (peripheral time) were recorded. The detailed methodology has been described elsewhere (Palenicek *et al.*, 2008, 2013).

Assessment of different behavioural patterns

Animals were housed separately in cages immediately following drug administration. Behavioural activity was evaluated (during the light phase of the cycle and under room lighting) in a 5-min open-field test (arena 68 × 51 × 35 cm). The arena, which was a novel environment, was placed in a soundproof and uniformly lit room. The behaviour of the rats was recorded using Activities software (script programmed in our laboratory). The following behavioural patterns (total number and time spent) were distinguished: (a) rearing, (b) grooming, (c) sniffing, (d) immobility, (e) flat body posture and (f) wet dog shakes (head twitch behaviour), as described by Palenicek *et al.* (2010).

Prepulse inhibition of the acoustic startle reaction

All of the rats were initially tested in a short session (a 5 min acclimatization period plus five single pulses) to determine the drug-free baseline startle magnitude 2 days before the experiment. On the day of the experiment, psilocin was administered, alone or with 5-HT antagonists or their respective vehicles, according to the scheme described above. All of the testing was performed in the startle chamber (SRLAB; San Diego Instruments, San Diego, California, USA). A high-frequency loudspeaker inside the chamber produced both a background noise of 62 dB and the 120 dB acoustic stimulus. The experimental procedure was adopted from

our previous studies (Palenicek *et al.*, 2008, 2013). After the acclimatization period the test began with five initial startle stimuli (120 dB) followed by four different trial types, presented in a pseudorandom order: (a) single pulse: 120 dB broadband burst, 20 ms duration; (b) prepulse: 13 dB, 20 ms duration above the background noise, presented 100 ms before the onset of the pulse alone; (c) prepulse alone: 13 dB, 20 ms duration above the background noise; (d) no stimulus. Five presentations of each trial type were given with an interstimulus interval of about 30 s. The PPI was expressed as a percentage [$100 - (\text{mean response for the prepulse-pulse trials} / \text{startle response for the single-pulse trials}) \times 100$]. The four single pulse trials at the beginning of the test session were not included in the calculation of the PPI values. Animals with a mean value lower than 10 were excluded from the calculation of the PPI acoustic startle reaction (ASR) and were considered as nonresponders. The number of nonresponders did not differ significantly between treatment groups.

Drugs

Psilocin and antagonists or their respective vehicles were administered at the same time 15 min before the behavioural testing in separate injections. All substances were administered subcutaneously in a volume of 2 ml/kg. Psilocin (*N,N*-dimethyltryptamine) (synthesized at the Pharmaceutical Faculty of Charles University in Prague) was dissolved in 2 ml of saline (0.9% NaCl), acidified with 10 µl of glacial acetic acid and subsequently adjusted to a final volume of 5 ml. The selective 5-HT_{1A} antagonist WAY100635 maleate (Sigma-Aldrich, Prague, Czech Republic) (Fornal *et al.*, 1996) and 5-HT_{2A} antagonist MDL100907 tartarate (ABX GmbH) (Herth *et al.*, 2009) were dissolved in saline. The selective 5-HT_{2B} antagonist SB215505 (free base) (Sigma-Aldrich) (Kantor *et al.*, 2004) was dissolved in 40% (2-hydroxypropyl)-β-cyclodextrine in saline with 25 mmol/l of citric acid, sonicated and then neutralized with NaOH to a pH of 7.4. The selective 5-HT_{2C} receptor antagonist SB242084 dihydrochloride (Tocris, Bristol, UK) (Kennett *et al.*, 1997) was dissolved in 10% (2-hydroxypropyl)-β-cyclodextrine in saline with 25 mmol/l of citric acid. All vehicles were tested and compared with saline, which served as a control. The doses used were as follows: psilocin 0.25, 1 and 4 mg/kg; WAY100635 maleate 1 mg/kg; MDL100907 tartarate 0.5 mg/kg; SB215505 1 mg/kg; and SB242084 1 mg/kg. Doses of antagonists were selected on the basis of previous animal studies (Steed *et al.*, 2011).

Statistical analysis

Statistical analysis of all data was performed using the programs Sigmapstat v. 3.0 (Systat Software Inc., San Jose, California, USA) and Statistica v. 9.0 (StaSoft Inc., Tulsa, Oklahoma, USA). Data from the experiments on sex differences were analysed with two-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc

tests if appropriate. When comparing the sexes, psilocin treatment and sex were used as factors. The same data set of male rats was used for analysis of sex differences as well as for analysis of experiments with serotonergic antagonists. When comparing the antagonists, 4 × 5 two-way factorial ANOVAs with the psilocin treatment (0, 0.25, 1, 4 mg/kg) and the antagonist treatment (vehicle and four antagonists) as the factors were used, followed by Newman-Keuls post-hoc tests. The level of significance was set as P less than 0.05. Data numerically distant by more than 2 SDs from the mean were excluded from the statistical analysis as outliers (0.3%). The number of outliers did not differ significantly among all treatment groups. After exclusion of PPI nonresponders and/or outliers, 8–12 animals remained in each experimental group for statistical analyses.

Results

Sex differences

Sex differences in locomotor activity

Two-way ANOVA showed significant effects of psilocin treatment [$F(3,107)=5.84$, $P<0.001$] and of sex [$F(2,107)=19.64$, $P<0.001$], and the sex × treatment interaction almost reached significance [$F(6,107)=2.01$, $P=0.071$]. Psilocin induced a dose-dependent decrease in locomotor activity in male and MD female rats but not in PE female rats. Significance was reached at 1 mg/kg psilocin in males and at 4 mg in both groups ($P<0.05$ and 0.01). While there were no significant differences between saline-treated animals, in general female rats were less sensitive to the locomotor inhibitory effects of psilocin than were males. This became apparent for all doses in PE females and for 1 and 4 mg/kg in MD females ($P<0.05$ –0.001) (Fig. 1).

The main effect of sex [$F(3,108)=12.36$, $P<0.001$] was the only significant effect in the analysis of time spent in the centre of the arena. Both groups of females spent more time in the centre than did males after psilocin treatment ($P<0.05$ –0.001) (data not shown). There were no significant changes in thigmotaxis.

Sex differences in qualitative behaviour

The two-way ANOVA analysis revealed a significant effect of treatment on all behavioural parameters [$F(3,129)=39.60$ (rearing), 22.53 (grooming), 15.26 (sniffing), 31.42 (immobility), 38.27 (flat body posture), 17.52 (wet dog shakes), $P<0.001$]. A significant effect of sex was observed for only one parameter, rearing [$F(3,129)=3.23$, $P<0.05$]. There was no significant sex × treatment interaction in any of the analyses, indicating no general sex differences.

Post-hoc analyses showed (Fig. 2) that psilocin, especially at the highest dose used, significantly attenuated the total time spent rearing, grooming and sniffing in all groups ($P<0.05$ –0.001). In contrast, the time spent in immobility and flat body posture significantly increased,

being most prominent at the highest dose ($P<0.05$ –0.001). The frequency of wet dog shakes increased after 0.25 mg/kg psilocin only in PE and MD female rats ($P<0.001$). The only isolated differences between sexes were observed for sniffing (both female groups spent more time sniffing than did males after 1 mg/kg psilocin: $P<0.05$) and wet dog shakes (both female groups showed more wet dog shakes than did males after 0.25 mg/kg psilocin: $P<0.01$ –0.001; data not shown).

Sex differences in PPI ASR

Two-way ANOVA revealed a significant effect of psilocin treatment on ASR [$F(3,121)=2.58$, $P<0.01$] but no significant effect of sex or interaction. Post-hoc tests did not identify any significant effect of psilocin treatment on ASR. An isolated difference between males and MD females was revealed by post-hoc tests: ASR in males was significantly lower compared with that in MD females ($P=0.05$) (Table 1).

The analysis of psilocin effects on PPI showed a borderline effect of psilocin treatment on PPI [$F(3,121)=2.58$, $P=0.056$], but no significant effect of sex or interaction. An additional one-way ANOVA in males only showed a clear effect of treatment [$F(3,38)=3.14$, $P<0.05$] with 1 mg/kg psilocin causing a disruption of PPI ($P<0.05$) (Fig. 3).

Effects of selective serotonin antagonists on psilocin-induced behavioural changes

Effect of the vehicle treatments

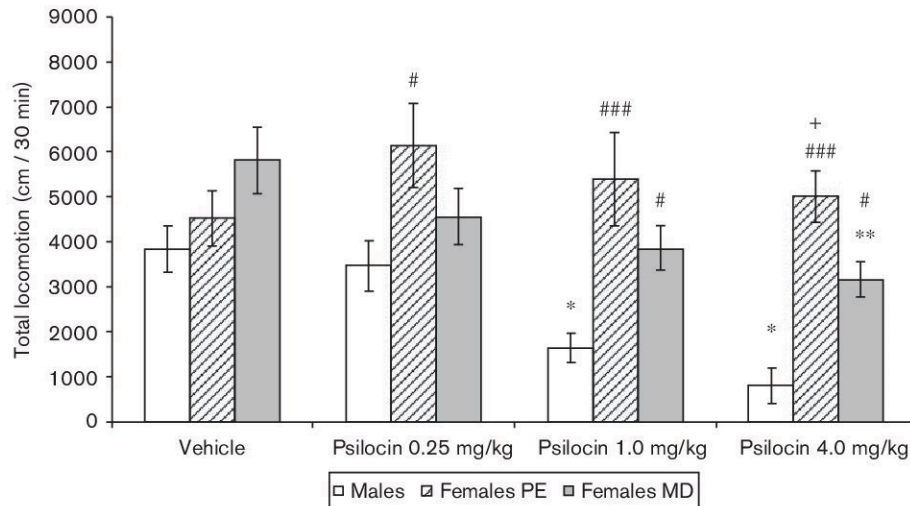
None of the vehicles had effects that were significantly different from those seen in saline-treated animals in any of the tests performed (data not shown).

Effects of antagonists on psilocin-induced changes in locomotor activity

Two-way ANOVA confirmed significant effects of the psilocin [$F(3,176)=11.62$, $P<0.001$] and antagonist [$F(4,176)=17.06$, $P<0.001$] treatments, and a significant interaction [$F(12,176)=2.565$, $P<0.01$].

Post-hoc analyses showed that WAY100635 and SB242084 restored the inhibition of locomotion by 1 and 4 mg/kg psilocin ($P<0.05$ –0.01). Administration of WAY100635 with 0.25 mg/kg psilocin yielded hyperlocomotion ($P<0.01$). SB215505 did not reverse the hypolocomotion significantly; however, the locomotor activity was not different from that of the control animals either. MDL100907 had no effect or slightly potentiated the locomotor inhibition induced by 1 and 4 mg/kg psilocin ($P<0.01$). Except for SB242084, which had a mild nonsignificant stimulatory effect, none of the other antagonists used had any significant effect on locomotor activity when administered alone (Fig. 4).

Fig. 1



The effect of psilocin on locomotor activity. Psilocin 0.25, 1 and 4 mg/kg dose-dependently decreased locomotor activity in male and MD female rats. ^{***} $P < 0.05$, 0.01 from the respective control group. ^{####} $P < 0.05$, 0.001 between males and females. ^{*} $P < 0.05$, 0.001 between pro-oestrus + oestrus (PE) females and metoestrus + dioestrus (MD) females.

Effects of antagonists on psilocin-induced changes in PPI ASR

Two-way ANOVA showed significant effects of psilocin [$F(3,185) = 5.32$, $P < 0.01$] and antagonist [$F(4,185) = 5.05$, $P < 0.001$] treatments on ASR, and a significant interaction [$F(12,185) = 2.03$, $P < 0.05$]. In the post-hoc tests, however, the only significant effect was observed for MDL100907 with 0.25 mg/kg psilocin, where ASR was significantly higher compared with 0.25 mg/kg psilocin and saline treatment ($P < 0.01$ – 0.001 in both cases) (Table 2).

Two-way ANOVA showed significant effects of psilocin [$F(3,185) = 5.54$, $P < 0.01$] and antagonist [$F(4,185) = 3.91$, $P < 0.01$] treatments on PPI, but no significant interaction. The only statistical trend in post-hoc tests supported a weak disruptive effect of 1 mg psilocin ($P = 0.08$) (Fig. 5).

Discussion

There are two main outcomes in our study. The first is that psilocin-induced behavioural effects were dependent not only on 5-HT_{2A} receptors, but 5-HT_{1A} and 5-HT_{2B/C} receptors also contribute significantly. The second important finding is that female rats, especially during the pro-oestrus and oestrus phases of the cycle, were affected by psilocin treatment to a lesser degree than were male rats. These findings are discussed below in the context of current knowledge about the function and role of the serotonin system and steroid-dependent regulatory mechanisms.

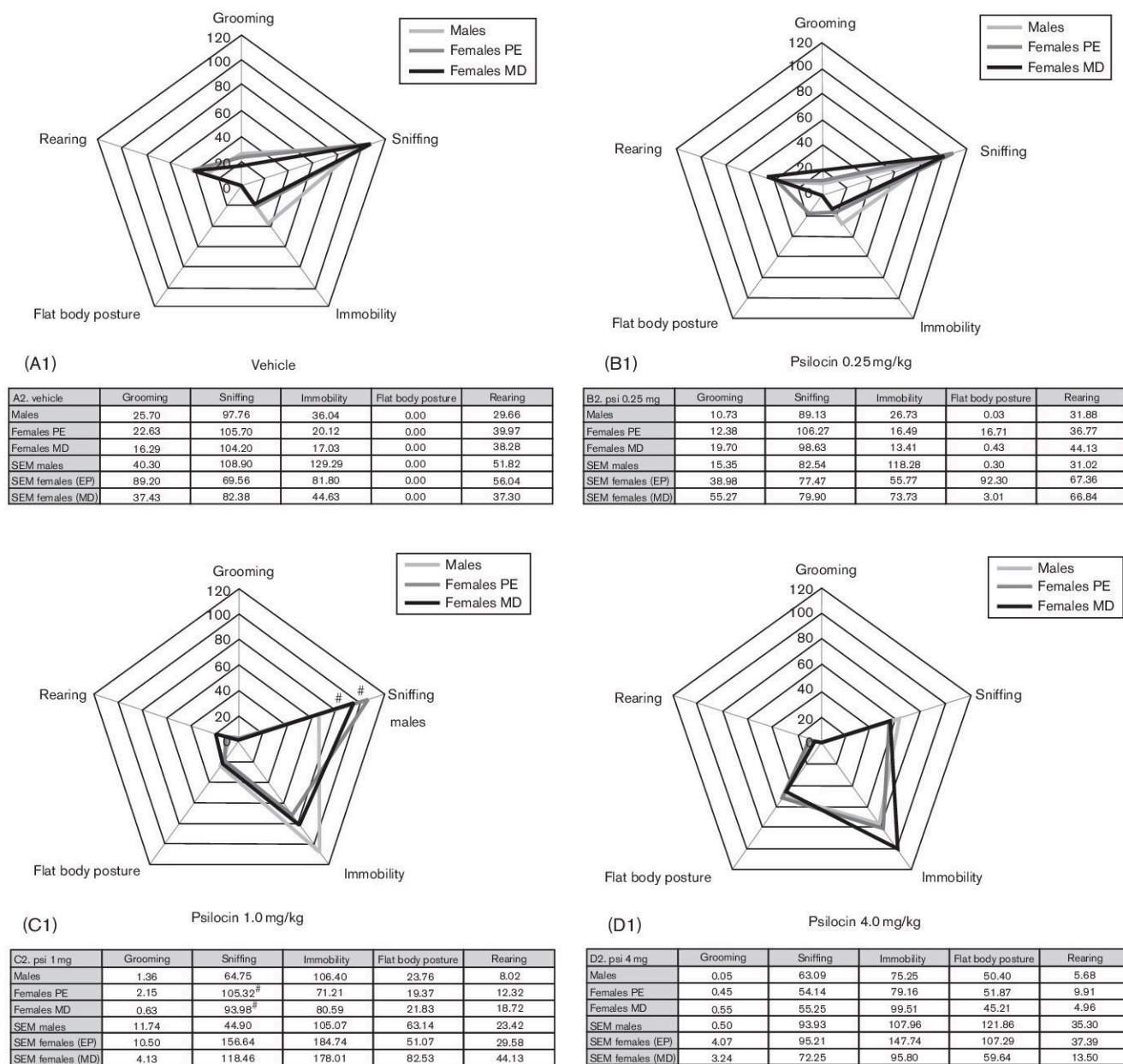
Behavioural effects of psilocin and the role of 5-HT_{1A} and 5-HT_{2A/B/C} receptors

In accordance with other studies with psychedelics (Palenicek *et al.*, 2008, 2010, 2013; Halberstadt and Geyer,

2011; Rambousek *et al.*, 2014) psilocin attenuated the normal behaviour of rats – that is, it induced strong sedation and/or ataxia, characterized by decreased locomotion, less time spent rearing, grooming and sniffing, and more time spent in immobility. Behavioural patterns typical for the serotonin syndrome, flat body posture and wet dog shakes, also occurred. We also found that psilocin disrupted PPI in male rats treated with 1 mg/kg psilocin and it tended to decrease the startle reaction. Even though we cannot directly measure the hallucinatory effects of psilocin, we can speculate that visual perceptual changes may also be present in rats, as they have been described in primates (Fantegrossi *et al.*, 2004). The observed behavioural changes might therefore simply reflect an altered perception of the environment. Furthermore, animals were tested during the light phase of their cycle, when they typically exhibit less locomotor and exploratory activity, which may also partly contribute to the observed behavioural inhibition. Our further investigation of the pharmacologic mechanisms revealed that 5-HT_{1A} and 5-HT_{2B/C} receptors are involved in these locomotor inhibitory effects. Even though the significant interaction between treatment factors supports an effect of 5-HT_{2C} antagonism on the action of psilocin, this should be interpreted cautiously, because we cannot totally exclude an additive effect of SB-242084 on stimulation of locomotor activity. On the other hand the disruption of PPI is mediated partially through 5-HT_{2A} and probably also to a lesser extent through 5-HT_{1A} receptors (these results were not significant).

Comparable results with various psychedelics and 5-HT_{1A} receptor antagonists have been also found in other behavioural studies (Krebs-Thomson and Geyer,

Fig. 2



Behavioural repertoire of the control animals in the open-field test. The data are shown as the total time in seconds (numbered axis) spent performing five behavioural patterns (the five axes of the pentagram) for vehicle-treated animals (A1), and psilocin 0.25, 1 and 4 mg/kg groups (B1, C1, D1). Psilocin significantly decreased sniffing, rearing and grooming in all animals, but flat body posture and immobility increased. The exact times in seconds (mean and SE) are shown in tables below the diagrams (A2–D2). [#] $P < 0.05$ between males and females. MD, metoestrus + dioestrus; PE, pro-oestrus + oestrus.

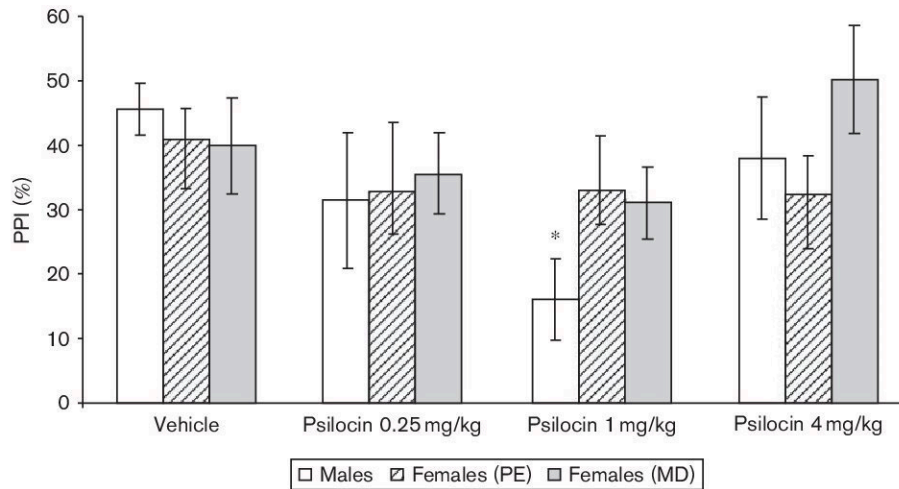
Table 1 The effect of psilocin on acoustic startle reaction amplitude in male and female rats

	ASR amplitude $\pm$ SEM			
	Vehicle	Psilocin 0.25 mg/kg	Psilocin 1 mg/kg	Psilocin 4 mg/kg
Males	79.92 $\pm$ 9.65	49.48 $\pm$ 11.62	56.30 $\pm$ 7.06	50.72 $\pm$ 10.89
Females (PE)	68.60 $\pm$ 9.32	68.64 $\pm$ 14.13	39.96 $\pm$ 7.37	54.70 $\pm$ 7.99
Females (MD)	74.41 $\pm$ 22.73	88.17 $\pm$ 10.59 [#]	44.68 $\pm$ 3.38	57.18 $\pm$ 3.92

ASR, acoustic startle reaction; MD, metoestrus + dioestrus; PE, pro-oestrus + oestrus.

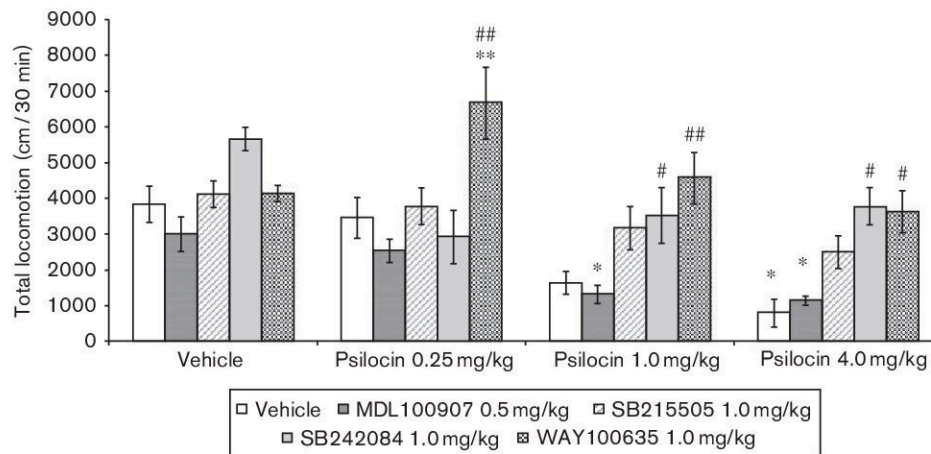
[#] $P < 0.05$ between male and female sex groups.

Fig. 3



The effect of psilocin on prepulse inhibition (PPI) of the acoustic startle response in male and female rats. Psilocin at the intermediate dose disrupted PPI in males. * $P < 0.05$ relative to vehicle. MD, metoestrus + dioestrus; PE, pro-oestrus + oestrus.

Fig. 4



The effect of serotonin receptor antagonists on psilocin-induced changes in locomotor activity. Inhibition of locomotion induced by psilocin was reversed by a 5-HT_{2C} antagonist (SB242084) and by a 5-HT_{1A} antagonist (WAY100635). *, ** $P < 0.05$ –0.01 for differences from saline-treated animals (vehicle-vehicle group); #, ## $P < 0.05$ –0.01 for significant effect of an antagonist within each psilocin treatment condition.

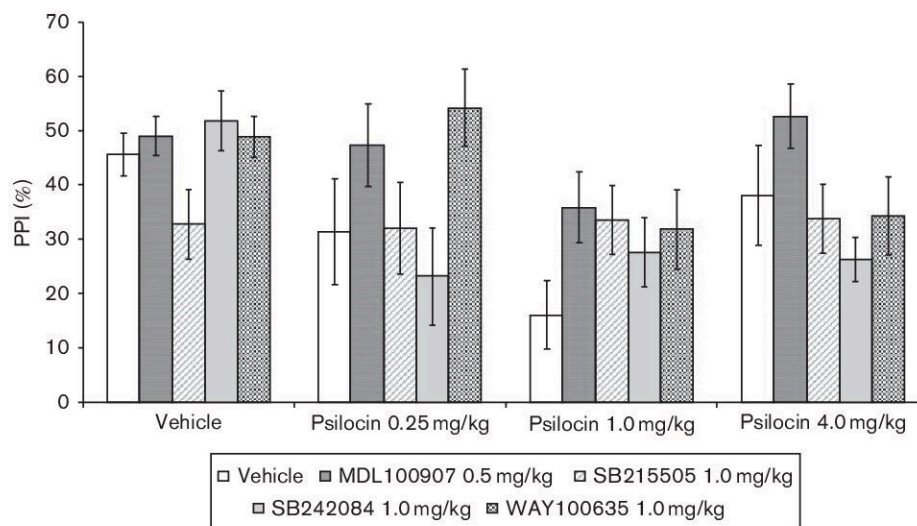
Table 2 The effect of psilocin and antagonist treatment on acoustic startle reaction amplitude

	ASR amplitude $\pm$ SEM			
	Vehicle	Psilocin 0.25 mg/kg	Psilocin 1 mg/kg	Psilocin 4 mg/kg
Vehicle	79.92 $\pm$ 9.65	49.48 $\pm$ 10.40	56.30 $\pm$ 7.06	50.72 $\pm$ 9.76
MDL100907 0.5 mg/kg	83.55 $\pm$ 8.47	160.53 $\pm$ 34.28***	101.50 $\pm$ 11.08	64.69 $\pm$ 7.85
SB215505 1.0 mg/kg	72.42 $\pm$ 7.86	98.22 $\pm$ 30.64	69.82 $\pm$ 8.98	59.20 $\pm$ 10.26
SB242084 1.0 mg/kg	88.24 $\pm$ 23.09	82.18 $\pm$ 10.47	53.72 $\pm$ 8.28	63.40 $\pm$ 6.12
WAY100635 1.0 mg/kg	66.64 $\pm$ 5.99	83.04 $\pm$ 11.30	61.78 $\pm$ 6.13	62.64 $\pm$ 8.46

ASR, acoustic startle reaction.

** $P < 0.01$ indicates differences from saline-treated animals (vehicle-vehicle group); *** $P < 0.001$ – indicates a significant effect of an antagonist within each psilocin treatment.

Fig. 5



Lack of effect of serotonin receptor antagonists on psilocin-induced disruption of prepulse inhibition (PPI).

1996; Halberstadt *et al.*, 2011). There are two main subtypes of 5-HT_{1A} receptors, presynaptic and postsynaptic, each of them having a different role. Whereas the presynaptic receptors serve as autoreceptors and control serotonin release in the raphe nuclei, postsynaptic receptors are located mainly in cortical pyramidal neurons and in hippocampal pyramidal and granular neurons (Barnes and Sharp, 1999). 5-HT_{1A} autoreceptors are more sensitive (almost five-fold) to indolamines compared with postsynaptic 5-HT_{1A} receptors. Thus, indoleamines induce an overall decrease in serotonergic tonic activity in the brain (Aghajanian and Hailgler, 1975; Nichols, 2004; Halberstadt and Geyer, 2011; Tyls *et al.*, 2014). Decreased serotonin tone is typically associated with locomotor inhibition, which can in turn be antagonized by agonism at 5-HT_{1A} postsynaptic receptors (Ahlenius and Salmi, 1995). Congruently, 5-HT_{1A} agonism at postsynaptic receptors has been shown to induce some signs of the behavioural serotonin syndrome (e.g. head waving, forepaw treading or flat body posture) (Smith and Peroutka, 1986; Sanchez *et al.*, 1996; Halberstadt and Geyer, 2011). From our results with a 5-HT_{1A} antagonist, we are able to conclude that locomotor inhibition and most likely also flat body posture are mediated by agonistic activity of psilocin at 5-HT_{1A} receptors.

However, locomotor inhibition induced by psilocin can also be related to its effects on 5-HT_{2B/C} but not 5-HT_{2A} receptors. The finding that 5-HT_{2A/C} receptors have an opposite role in the control of locomotor activity has been previously described (Halberstadt *et al.*, 2009). A plausible explanation is related to dopamine, a crucial neurotransmitter involved in motor control. Whereas stimulation of 5-HT_{2A} receptors induces a phasic increase in the

release of dopamine, stimulation of 5-HT_{2C} receptors decreases the tonic activity of the dopaminergic system (Barnes and Sharp, 1999; Lucas and Spampinato, 2000). Therefore, psilocin, through agonist activity at 5-HT_{2C} receptors, would also decrease the tonus of the dopaminergic system, which may result in locomotor inhibition and ataxia. A 5-HT_{2C} antagonist, in turn, would increase dopaminergic activity, resulting in normalization of locomotion. The new finding of this study was that a 5-HT_{2B} antagonist normalized locomotor inhibition to a level comparable to that produced by a 5-HT_{2C} antagonist. Despite the fact that little is known about the role of 5-HT_{2B} receptors, psilocin has a comparable or higher affinity to this receptor subtype than to 5-HT_{2A/C} receptors (Ray, 2010; Tyls *et al.*, 2014). 5-HT_{2B} receptors are found in the rat brain in the cerebellum (Purkinje cells), lateral septum, dorsal hypothalamus and medial amygdala (Barnes and Sharp, 1999). Therefore, we hypothesize that psilocin might contribute to ataxia by acting on this receptor subtype in Purkinje cells, and that the antagonist reversed these effects. One might speculate about the selectivity of the 5-HT_{2B/C} antagonists used. SB215505 has been previously reported to have a 95-fold higher selectivity at 5-HT_{2B} over the 5-HT_{2C} subtype (Kantor *et al.*, 2004). However, in another study only a moderate selectivity over 5-HT_{2C} receptors was reported (Reavill *et al.*, 1999). In contrast, SB242084 has a 100- and 158-fold selectivity at 5-HT_{2B} and 5-HT_{2A} subtypes (Kennett *et al.*, 1997). Taken together we cannot exclude a minor contribution of 5-HT_{2C} antagonism in the effects of SB215505.

Interestingly, the blockade of the 5-HT_{1A} receptor with the lowest psilocin dose induced hyperlocomotion, an

effect typically associated with a hyperdopaminergic state (Palenicek *et al.*, 2005). In line with this, psilocin has been shown to induce dopamine release in the striatum in humans (Vollenweider *et al.*, 1999) and, more recently, also in the nucleus accumbens of male Wistar rats, although only with a very high dose of 10 mg/kg of psilocin (Sakashita *et al.*, 2015). One plausible explanation is that a 5-HT_{1A} antagonist could unmask less significant agonism on 5-HT_{1B/D} receptors (Tyliš *et al.*, 2014). Activation of these receptors has been shown to increase the dopamine release in the mesolimbic pathway (Yan *et al.*, 2005) and the antagonists are also able to block hyperlocomotion induced by the 5-HT/dopamine releaser methylendioxyamphetamine (McCreary *et al.*, 1999; Bankson and Cunningham, 2002). The lack of this stimulating effect at higher doses could be related to the effects of psilocin on 5-HT_{2C} receptors, which are well known to inhibit locomotion and dopamine release when activated (Barnes and Sharp, 1999; Lucas and Spampinato, 2000; Palenicek *et al.*, 2008, 2013).

The psilocin-induced changes in sensorimotor gating were statistically significant only when comparing male and female rats, where only 1 mg/kg psilocin disrupted PPI in male rats. Even though the magnitude of PPI after 1 mg/kg psilocin was 35% of that of control animals, this isolated effect did not survive multiple comparisons of the analysis with antagonists. However, the administration of a 5-HT_{2A} antagonist yielded a PPI magnitude of 79% that of control animals, and similarly 5-HT_{1A} and 5-HT_{2B/C} antagonists increased PPI to 60–74% of control values. Therefore, we might assume that a tendency to normalize the effects was present for all antagonists and most remarkably for the 5-HT_{2A} antagonist. This is in accordance with other studies on hallucinogens, where the same 5-HT_{2A}-dependent mechanisms have been described in animals (Sipes and Geyer, 1995b; Ouagazzal *et al.*, 2001; Halberstadt and Geyer, 2010) and in the case of psilocybin also in humans (Quednow *et al.*, 2012). Differently from other hallucinogens, we have also observed that a PPI deficit was not present with antagonists of 5-HT_{1A} or 5-HT_{2B/C} receptors. Interestingly, a PPI deficit induced by the structurally related tryptamine hallucinogen 5-MeO-DMT was congruently blocked by 5-HT_{1A} and 5-HT_{2B/C} but not by the 5-HT_{2A} antagonist (Krebs-Thomson *et al.*, 2006). The role of 5-HT_{1A} receptors in the effect of psilocin on PPI is further supported by the fact that 5-HT_{1A} agonists decrease PPI in rats (Rigdon and Weatherspoon, 1992; Sipes and Geyer, 1995a). Therefore, we cannot exclude a minor contribution of these receptors in the PPI-disruptive effect of psilocin.

Two interesting findings, the biphasic effect, characterized by the lack of the PPI-disruptive effect and wet dog shakes at the higher psilocin dose, deserve further discussion. Such a biphasic effect has also been described for other behavioural parameters induced by hallucinogens, especially

locomotor activity (Davies and Redfern, 1973; Fantegrossi *et al.*, 2008; Palenicek *et al.*, 2008). The effect on wet dog shakes was also found in other studies where psilocin increased this particular behavioural pattern only up to a dose of 2.4 mg/kg (Vickers *et al.*, 2001; Halberstadt *et al.*, 2011). As both of these mechanisms are typically related to 5-HT_{2A} agonist activity, something that attenuates the 5-HT_{2A} response at the higher dose must be present. A mechanistic explanation can be related to the inhibition of motor functions and fits with the inhibition of locomotion and other behaviour. Another perspective comes from a study evaluating the role of the 5-HT_{2C} receptor in head twitching behaviour (wet dog shakes). This study reported that the 5-HT_{2C} antagonist triggers 5-HT_{2A}-mediated head twitch behaviour with less selective drugs, such as TFMPP (3-trifluoromethylphenylpiperazine) or mCPP (meta-chlorophenylpiperazine), that act predominantly as agonists at the 5-HT_{2C} receptor (Vickers *et al.*, 2001). Similarly, 5-HT_{2C} agonism also ameliorated a PPI deficit induced by DOI (2,5-dimethoxy-4-iodoamphetamine), a potent 5-HT_{2A/C} agonist (Marquis *et al.*, 2007). As psilocin is known to be a strong agonist at 5-HT_{2C} receptors (Tyliš *et al.*, 2014) this particular mechanism can in the same way mask both behavioural patterns at the higher psilocin dose.

Sex differences in behavioural effects and underlying mechanisms

Except for wet dog shakes, female rats were in general affected by psilocin to a lesser degree than were male rats. This finding is fully in line with our previous results on sex differences in the effects of LSD (Palenicek *et al.*, 2010). One plausible explanation of the observed sex differences can be related to the simple fact that females are naturally more explorative than males and should be naturally more attentive to their environment (Tropp and Markus, 2001; Brown and Nemes, 2008). In line with this hypothesis, female rats during the pro-oestrus and oestrus phase were slightly more active without the drug than were males (Fig. 1) and also spent more time exploring the centre of the arena. An alternative interpretation can be derived from early studies showing that female steroids protect rats from dyskinesia, inhibition of climbing and other behavioural patterns induced by LSD (Bergen *et al.*, 1960; Selye, 1971). Progesterone and related steroids have been reported to attenuate some effects of LSD in humans (Krus *et al.*, 1961, 1967). As female hormones are at their highest levels during the pro-oestrus and oestrus phases (Smith, 1994), it can be assumed that these hormones protect female rats from the disruptive effects of psilocin in our setting.

The prominent role of steroid hormones in the modulation of the serotonin system is well known. Sexual dimorphism has been described in basal and stimulated serotonin levels, with females showing higher levels of serotonin, compared with males, in various brain regions [for a review see Rubinow *et al.* (1998)], including the

dorsal raphe nucleus (Dominguez *et al.*, 2003). During the pro-oestrus and oestrus phase, most findings congruently describe higher levels of serotonin (Rubinow *et al.*, 1998). In addition, the response to a 5-HT_{1A} agonist is in general decreased during the pro-oestrus and oestrus phases (Rubinow *et al.*, 1998). Most authors also report that oestrogens decrease 5-HT_{1A} mRNA expression and the number of these receptors (e.g. Osterlund *et al.*, 1999, 2000; LeSaux and DiPaolo, 2005), including the auto-receptors in the dorsal raphe nucleus (Uphouse *et al.*, 1991). The effect of steroids on the 5-HT_{2C} receptor is less clear (Gundlach *et al.*, 1999; Birzniece *et al.*, 2002; Zhou *et al.*, 2002) and we found no evidence of sex-dependent or steroid-dependent differences in the 5-HT_{2B} receptor. In contrast, the higher activity of the dopamine system in females, related to oestrogens and progesterone, has been well documented (Attali *et al.*, 1997; Becker and Rudick, 1999; Zhou *et al.*, 2002; Palenicek *et al.*, 2005). Taken together, females, especially during the pro-oestrus and oestrus phases, should be protected from the effects of 5-HT_{1A}-mediated changes and should have higher activity of the dopaminergic system, resulting in the blockade of the inhibitory effect on locomotion.

As stated above, the disruptive effects of hallucinogens on PPI are 5-HT_{2A} mediated. There is abundant evidence of an increased binding/expression of 5-HT_{2A} receptors in females and increased numbers of these receptors due to oestrogen treatment (Sumner and Fink, 1995, 1998; Osterlund *et al.*, 1999; Cyr *et al.*, 2000; Birzniece *et al.*, 2002; Sumner *et al.*, 2007). According to these studies, the bigger pool of these receptors in females, especially during the pro-oestrus and oestrus phases, may underlie the lower sensitivity of female rats to the disruptive effect on PPI [a higher drug dose would be necessary for sufficient receptor occupation, as we have hypothesized previously (Palenicek *et al.*, 2010)]. However, the ineffectiveness of 4 mg/kg psilocin to disrupt PPI in both sexes makes this simple explanation related to 5-HT_{2A} receptors less clear, and further experiments would be needed to elucidate the mechanisms.

Conclusion

Psilocin showed a similar behavioural profile to other hallucinogens. Apart from the well-known effects mediated through serotonin 5-HT_{2A} receptors, our study showed an important contribution of 5-HT_{1A} and 5-HT_{2B/C} receptors to its behavioural action. We have also confirmed our previous finding with LSD (Palenicek *et al.*, 2010) that female rats, especially during the pro-oestrus and oestrus phases, are protected from some of the effects of hallucinogens. Even though in humans no sex differences have been found in responses to psilocybin (Studerus *et al.*, 2012), our recent results, as well as previous human studies with LSD (Krus *et al.*, 1961, 1967), indicate that these might still be hidden or previously underexplored.

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Conflicts of interest

There are no conflicts of interest.

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