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PRENATAL EXPOSURE TO MODAFINIL ALTERS LOCOMOTOR BEHAVIOUR AND LEUCOCYTE PHAGOCYTOSIS IN MICE

Jana Ruda-Kucerova[1], Petra Amchova[1], Alena Machalova[1], Jana Pistovcakova[1] & Alexandra Sulcova[2]

[1]Department of Pharmacology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[2]Behavioral and Social Neuroscience Group, CEITEC - Central European Institute of Technology, Masaryk University, Brno, Czech Republic

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SUMMARY

Background: *Modafinil is a psychostimulant drug prescribed mainly for treatment of narcolepsy but is used as a "smart drug" by wide populations to increase wakefulness, concentration and overall mental performance. The aim of this study was to assess potential developmental toxicity of modafinil.*

Materials and methods: *Pregnant female mice were given either saline or modafinil (50 mg/kg orally) from gestational day (GD) 3 to GD 10 and then a challenge dose on the GD 17. The male offspring were treated analogously at the age of 10 weeks. Changes in the spontaneous locomotor/exploratory behaviour and anxiogenic profile in the open-field test were assessed in naïve animals, after an acute and 8th modafinil dose and the challenge dose following a 7-day wash-out period. One month after completion of the behavioural study, the leukocyte phagocytosis was examined by zymosan induced and luminol-aided chemiluminiscence assay in vitro.*

Results: *The most important finding of this study was the immunosuppressing effect on leukocyte activity, hypolocomotion and increased behavioural response to modafinil-induced psychostimulation caused by prenatal exposure to the same drug. We did not detect significantly altered anxiety-related behaviour in any group disregarding the pre- and postnatal treatments.*

Conclusion: *This is the first evidence of developmental toxicity of modafinil which needs to be taken into account as a potential risk factor when modafinil is administered to women who may become or are pregnant.*

Key words: *modafinil - prenatal administration – locomotion – phagocytosis - mice*

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INTRODUCTION

Modafinil is a psychostimulant drug used mainly for treatment of narcolepsy (Golicki et al. 2010), despite other indications have been proposed, e.g. as cognitive enhancer or substance abuse treatment (Mereu et al. 2013). The drug is well-tolerated and can be prescribed as a treatment of fatigue associated with AIDS (Rabkin et al. 2010) or multiple sclerosis (Tullman 2013) as well as in attention deficit hyperactivity disorder (ADHD) in children and adolescents (Wood et al. 2014). Furthermore, modafinil is used as a "smart drug" by wide populations (Vargo & Petroczi 2016) to increase wakefulness, concentration and overall mental performance (Wood et al. 2014).

The mechanism of action is complex and probably involves numerous neurotransmitter systems. The main effect is apparently exerted via blockade of domaminergic transporter (DAT), preventing re-uptake of dopamine (DA) back to the presynaptic neuron. This effect is analogous to cocaine but weaker. Despite modafinil was shown to exert addiction (Volkow et al. 2009) it is considered a medication with low abuse potential, but the regular use is not without risk (Wisor 2013).

In preclinical studies modafinil is known to cause a robust hyperlocomotion in rodents comparable with the effect of amphetamine or methamphetamine (Simon et al. 1995). Modafinil was shown to increase locomotor activity in a dose dependent manner similarly as MDMA (3,4-methylenedioxymethamphetamine, "ecstasy") or methamphetamine but had a different ethological profile in the mouse model of agonistic behaviour. At lower than psychostimulant doses modafinil produced anxiolytic-like and antiaggressive-like effects (Machalova et al. 2010a).

On comparison with methamphetamine and MDMA which produced dose-dependent inhibition of aggression at least some doses of modafinil increased aggression and decreased timidity with no effect on sociability (Machalova et al. 2012). Also development of dependence was reported in mice although at a high (125 mg/kg) dose (Nguyen et al. 2011). Furthermore, D1 receptor appears to exert a primary role in modafinil-induced effects on spontaneous exploration as this effect was abolished in the D1 knock-out mice (Young et al. 2011) while D2 agonistic profile contributes to its antidepressant-like properties recently reported in a mouse model (Mahmoudi et al. 2015). Similarly as cocaine or amphetamine-like psychostimulants modafinil was shown to exert behavioural sensitization (Paterson et al. 2010, Slais et al. 2010), a phenomenon described as increased behavioural response (usually locomotor) to a repeated intermittent administration of a stable dose of addictive substance (Robinson 1984, Boutrel 2008, Watterson et al. 2016). This phenomenon may even lead to decreased drug consumption (Kucerova et al. 2009, Kucerova et al. 2012). Behavioural sensitization to drugs of abuse and the related adaptations in striatal particularly

dopaminergic neurotransmission, are thought to play an important role in certain aspects of addiction such as tendency to relapse following abrupt drug withdrawal (Ohmori et al. 2000, Shuto et al. 2008). Pre-clinical studies use a variety of paradigms to exert this effect but they all assess the locomotor-exploratory activity at basal conditions before any treatment, after an acute drug exposure, then after a chronic treatment and lastly following a challenge dose after a period of wash-out (Landa et al. 2006, Landa et al. 2008, Paterson et al. 2010). Acute dose of a psychostimulant leads to increased locomotion and further increase after chronic exposure to the drug is considered development of sensitization. Equally high or higher locomotor response to a challenge dose is supposed to reflect expression of behavioural sensitization known to be present long after the drug discontinuation (Landa et al. 2014). In case of modafinil, the development of the sensitization was not shown but the expression after a challenge dose was observed (Paterson et al. 2010) and it is also able to induce a cross-sensitization to cocaine, i.e. increased locomotor response to a cocaine challenge dose after a chronic treatment with modafinil (Wuo-Silva et al. 2011). Modafinil as a wake-promoting agent was hypothesized to possess certain immunosuppressant effects analogously as a lack of sleep especially in patients using this drug for other than narcoleptic condition. Furthermore, a preliminary evidence shows an increase of C-reactive protein after an acute modafinil dose (Kim 2012). However, the immunomodulatory properties of modafinil have not been described in detail.

Prevalence of illicit drug use in pregnant women ranges around 4% and non-medical use of less harmful substances is expected to be even higher (Holbrook & Rayburn 2014). Due to modafinil use as a "smart drug" (Vargo & Petroczi 2016), the risk of its use during pregnancy is significant. Therefore, the aim of this study was to combine assessment of potential changes in the spontaneous locomotor/exploratory behaviour and anxiogenic profile in the open-field test in mice after prenatal modafinil exposure. For assessment of cell immune functions measurement of leukocyte phagocytic activity was selected (Pavelkova & Kubala 2004). Furthermore, postnatal exposure to modafinil was evaluated in both prenatally exposed and control group and possible development of behavioural sensitization was estimated. This study may provide evidence for potentially harmful effects of modafinil when taken by pregnant women. This possibility is likely due to modafinil use for cognitive enhancing effects and moderate psychostimulation (Wood et al. 2014, Vargo & Petroczi 2016).

MATERIALS AND METHODS

Animals

Adult male and female albino ICR mice were purchased from Masaryk University breeding facility and harem housed in cages of 5 females and 1 male (total n=30 females and 6 males). Day 4 of the harem housing was determined as gestational day 1 (GD 1). Mice were treated by either saline (SAL, 10 ml/kg, n=8) or modafinil (50 mg/kg, n=7) from GD 3 to GD 10 and then given a challenge dose on the GD 17 (see Table 1). This schedule was repeatedly found to induce behavioural sensitization to various psychostimulants (Landa et al. 2006, Paterson et al. 2010, Landa et al. 2011, 2012). The average surviving litter size was n=9.5 in control and n=10.1 in modafinil treated mothers. No cross-fostering was used, the mothers were regularly weighted and no differences were observed between control and modafinil treated mothers. The male offspring were weaned on the postnatal day (PND) 22 and housed in cages of 5. The behavioural testing started at age 10 weeks, specifically on PND 70. The drug dosage regimen was used in the adult male offspring was analogous to the one used in mothers, known to induce behavioural sensitization (see Table 1). Thus, there were four experimental groups (n=12 each): SAL and MDF offspring from SAL mothers and SAL and MDF offspring from MDF treated mothers. The animals were randomly assigned to the treatment groups assuring there will be no more than 2 subjects from the same litter in every group to avoid litter effects (Holson & Pearce 1992). Environmental conditions during the whole study were constant: relative humidity 50-60%, temperature 23ºC ±1ºC, normal 12-hour light-dark cycle (7 a.m. to 7 p.m. light). Food and water were available ad libitum. All procedures were performed in accordance with EU Directive no. 2010/63/EU and approved by the Animal Care Committee of the Faculty of Medicine, Masaryk University, Czech Republic and Czech Governmental Animal Care Committee, in compliance with Czech Animal Protection Act No. 246/1992.

Drugs and treatments

Modafinil (MDF) was isolated from a ready-made preparation (Vigil tbl. 100 mg) with 99.97% purity (HPLC) at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Charles University, Czech Republic. The solution for oral gavage was prepared by suspending 50 mg of MDF in 10 ml of saline with 0.5% gum arabic. Care was taken to shake the suspension before use and administer a highly homogeneous material. The dose administered was 50 mg/kg at the same time in the morning hours. On the days when open-field testing was performed the administrations were done 30 minutes before the start of the test. Saline was administered as vehicle to the control groups. The oral treatment was selected due to the necessity of chronic administration to pregnant dams. The dose choice was based on the documented high sensitivity of mice (compared to rats) to modafinil (Simon et al. 1996) and behavioural effect observed after oral administration of this dose in mice (Okuro et al. 2010). We have also observed a highly significant locomotor stimulation in the mothers after an acute dose, chronic dose and the challenge dose (data not shown).

Table 1. Study design - show treatment schedule used in mothers and their male offspring

Mothers:	GD 3	GD 4	GD 5	GD 6	GD 7	GD 8	GD 9	GD 10	GD 11	GD 12	GD 13	GD 14	GD 15	GD 16	GD 17
SAL (n=15)	saline orally (SAL)								No applications						SAL
MDF (n=15)	modafinil orally (MDF)								No applications						MDF

Male offspring: prenatal-postnatal treatment	PND 70	PND 71	PND 72	PND 73	PND 74	PND 75	PND 76	PND 77	PND 78	PND 79	PND 80	PND 81	PND 82	PND 83	PND 84	PND 85	PND 86	PND 87	PND 88	PND 89	PND 90	PND 91
SAL-SAL (n=12)	No applications							saline orally (SAL)								No applications						SAL
SAL-MDF (n=12)								modafinil orally (MDF)														MDF
MDF-SAL (n=12)								saline orally (SAL)														SAL
MDF-MDF (n=12)								modafinil orally (MDF)														MDF
Test	OF							OF							OF							OF

GD: gestational day; PND: postnatal day; SAL: saline treatment; MDF: modafinil treatment; OF: open-field test

Locomotor activity test

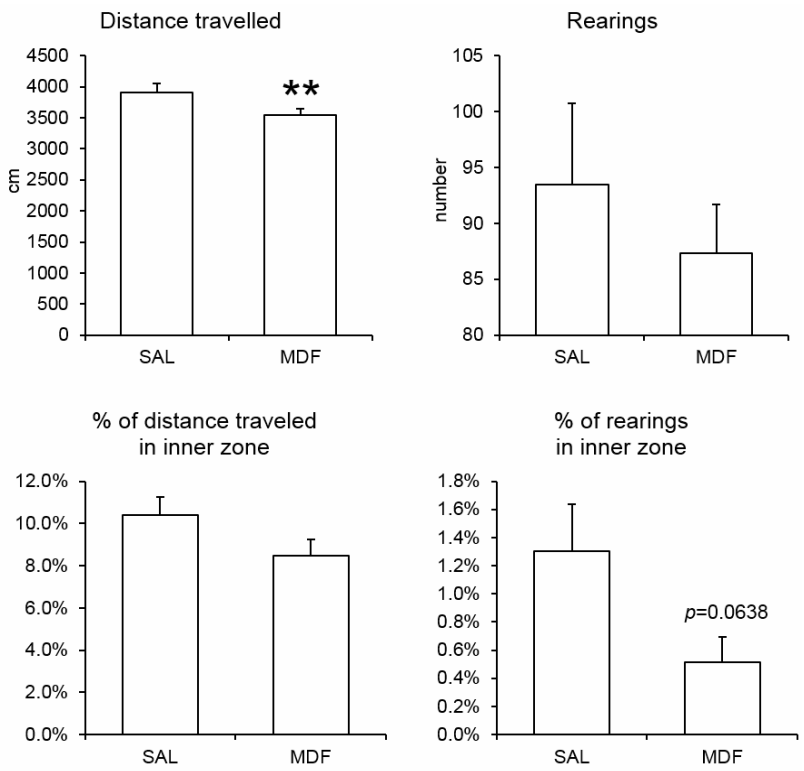
In brightly lit room, mice were individually tested for locomotor activity using the Actitrack system (Panlab, Spain) as previously described (Pistovcakova et al. 2008, Ruda-Kucerova et al. 2015, Ruda-Kucerova et al. 2016). Each plexiglass arena (45×45×30 cm) was surrounded by 2 frames equipped with photocells located one above another at 2 and 7 cm over the cage floor. Animals were placed individually in the centre of arena and the spontaneous horizontal (distance travelled) and vertical (rearing behaviour) locomotor activity was tracked automatically. At the end of the session, animals were returned to their home cage and the arenas were cleaned to remove potential olfactory cues. Locomotor activity (distance travelled) was recorded in the open field test for 9 minutes as follows: PND 70 – naïve mice, PND 77 – acute dose of SAL or MDF, PND 84 – one week repeated administration, PND 91 – challenge dose, i.e. acute dose after one week wash-out period. The main variables assessed were the total distance travelled as a measure of horizontal locomotion and the number of rearing episodes indicating the vertical exploratory activity. In order to assess also anxiogenic behaviour the proportion of both distance travelled and the number of rearings which took place in the central part of the arena were calculated. The central part of the arena was defined by a 10 cm peripheral margin from all edges.

The leukocyte phagocytosis assay

The mice were sacrificed by decapitation in short inhalation anaesthesia and trunk blood was collected into hearinized tubes. The animals were sacrificed on PND 120, i.e. one month after completion of behavioural experiment, in order to evaluate long-lasting effects of the treatments after the drug is washed-out from the system. The leukocyte phagocytosis was examined by zymosan induced and luminol-aided chemiluminiscence assay in vitro following a standard technique as described earlier (Pistovcakova et al. 2008). Briefly, 20 µl of whole blood were mixed with 500 µl of Hank's solution. A 200 µl sample was pipetted in a cuvette and 40 µl of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich, s.r.o., Prague, Czech Republic) at a concentration of 1.7 mg/ml was added. After a 10 min measurement of the "background chemiluminiscence" by the chemiluminometer (Biolumat LB 9500C, Berthold Co., Germany), phagocytosis was stimulated by addition of 40 µl of opsonized zymosan. The relative degree of specific phagocytosis was calculated by extrapolating data from the standard curve and by subtracting the values for the non-specific "background". The stimulated chemiluminescence was measured at 5 min intervals during one hour. The temperature was maintained at 37°C throughout the whole procedure. For the measurement of systemic leukocyte counts, 20 µl of blood were mixed with 10 ml of Isotonac 3 of diluent solution in a cuvette, and 6 drops of hemolyzing reagent (Medista, s.r.o., Czech Republic) were added and mixed. One minute later, the total leukocyte count was obtained using a semi-automatic haematology analyzer MEK-5208 K (Nihon Kohden, Tokyo, Japan). For differential white blood cell counts, the blood smears were prepared immediately after blood collection using a standard coverslip technique, and were air-dried and stained with Leukodif 200 set (Bio-La-Test, Pliva-Lachema a.s., Brno, Czech Republic). Differential leukocyte counts were assessed with the help of Leukomat 5XP 83202 (Tesla Kolin k.p., Czech Republic).

Statistical Data analysis

Primary data were summarized using arithmetic mean and standard error of the mean (±SEM) estimate and Kolmogorov-Smirnov test of normality was performed indicating some of the behavioural data as non-parametric. Therefore, locomotor data were analysed in every time-point by the Kruskal-Wallis ANOVA followed by multiple comparisons with Bonferroni correction. Despite some positive results of the normality test, we calculated the development of the behavioural profile by repeated measures ANOVA (factors: prenatal and postnatal treatment, repeated factor: measurement)



Graphs show variables measured in the open-field. The postnatal treatment was not initated at this time-point yet, therefore the animals with the same prenatal exposure are pooled (n=24 per group). Mann-Whitney U test revealed significantly lower distance travelled in the OF in MDF prenatally treated mice (**p≤0.01) and a weak trend towards decrease in rearing behaviour in the inner zone of the arena in the same group. All data are shown as means ±SEM.

Figure 1. Open-field in drug-naive animals

with Bonferroni post-hoc test, because there is no non-parametric equivalent for this comparison. The data sphericity was assessed by Mauchly test which confirmed that this assumption was not violated for any factor or interaction. The leucocyte chemiluminiscence was also analysed by repeated measures ANOVA (same factors). The analyses were calculated using Statistica 12 (StatSoft, USA). A value $p<0.05$ was recognized as boundary of statistical significance in all applied tests.

RESULTS

Basal open-field (PND 70)

The basal locomotor characteristics depicted in the Figure 1 show a significant decrease of horizontal locomotion induced by the prenatal MDF exposure (Mann-Whitney U test, p=0.005). Furthermore, the proportion of rearing episodes in the central zone shows in the MDF exposed mice a trend to a decrease (Mann-Whitney U test, p=0.064) suggesting a possibility of higher anxiety. In this time-point the mice were pooled depending on the prenatal exposure only.

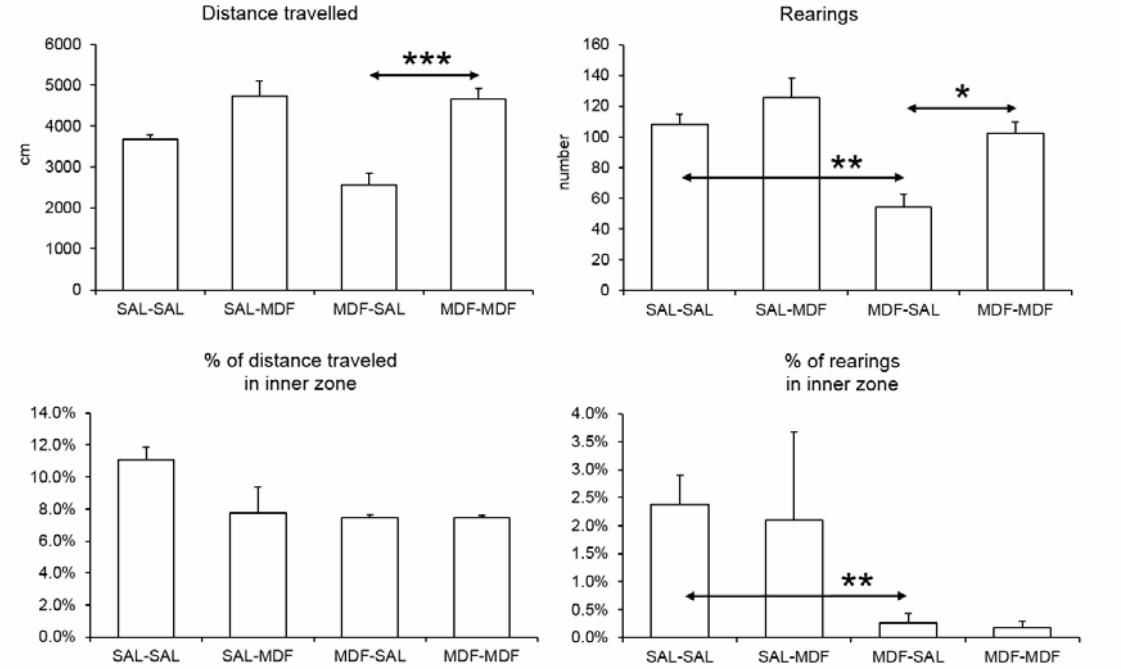
Open-field after acute dose of MDF (PND 77)

Figure 2 pools the data obtained after the acute administration of MDF or vehicle. In this time-point the mice were already divided into 4 groups by the pre- and postnatal treatment. Interestingly, MDF did not exert a significant effect on the control animals while there was a strong stimulation in the MDF prenatally exposed group. Specifically: Kruskal-Wallis ANOVA (KW ANOVA) identified significant differences between the groups: $H(3)=22.092$, $p<0.001$, and test for multiple comparisons revealed a significant increase of distance travelled in the MDF-MDF mice compared with MDF-SAL ($p<0.001$) while there was no such effect detected in the comparison of SAL-MDF and SAL-SAL mice (p=0.265). A similar outcome was obtained in the rearing behaviour: KW ANOVA: $H(3)=20.735$, $p<0.001$, in multiple comparisons MDF-MDF vs. MDF-SAL rendered p=0.042. Interestingly the hypoactivity of the MDF-SAL animals (compared to SAL-SAL) was again visible in this measure: p=0.006. No significant difference was found in the horizontal locomotion in the central zone of the arena, only the hypoactivity of the MDF-SAL animals was present analogously as in the total rearings: KW ANOVA: $H(3)=21.045$, $p<0.001$, multiple comparisons: MDF-SAL compared to SAL-SAL, p=0.003.

Open-field after chronic exposure to MDF (PND 84)

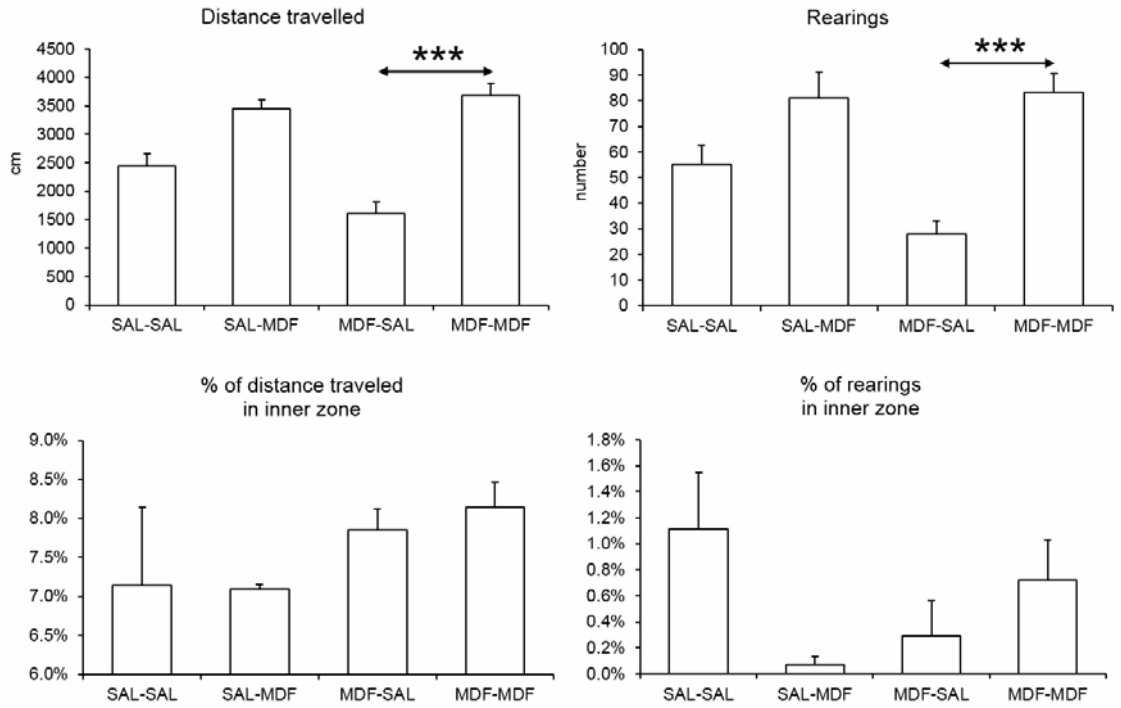
Chronic administration of MDF exerted similar behavioural effect as the acute one (Figure 3), i.e. higher stimulating effect on MDF prenatally exposed mice

than the control animals. Distance travelled: KW ANOVA H(3)=28.621, p<0.001, and test for multiple comparisons detected a significant increase of distance travelled in the MDF-MDF mice compared to MDF-SAL (p<0.001). A similar outcome was obtained in the number of rearings, KW ANOVA H(3)=21.470, p<0.001, MDF-MDF compared to MDF-SAL, p<0.001. No differences among the groups were found in the central zone.



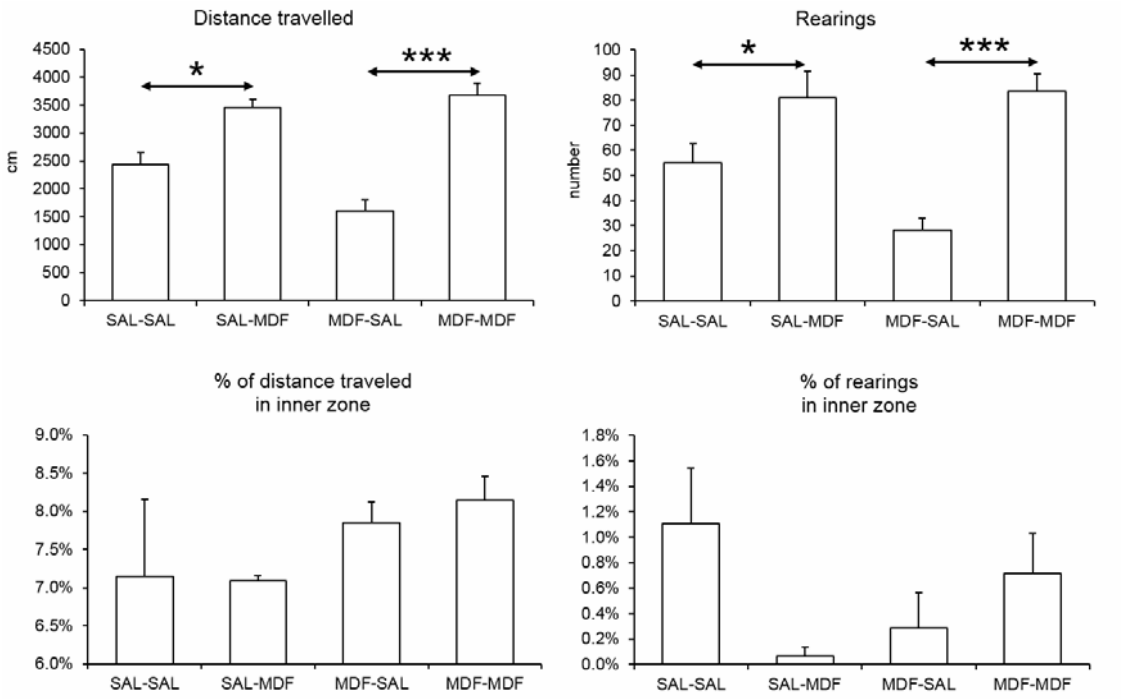
Kruskal-Wallis ANOVA indicated significant stimulating effect of MDF on both distance travelled and rearings in the MDF prenatally exposed animals only. The locomotion suppressingg effect of prenatal MDF treatment was still visible in the rearing behaviour. All data are shown as means ±SEM, n=12 per group; *p≤0.05; **p≤0.01; ***p≤0.001

Figure 2. Open-field after acute treatment



Kruskal-Wallis ANOVA indicated significant stimulating effect of MDF on both distance travelled and rearings only in the MDF prenatally exposed mice. All data are shown as means ±SEM, n=12 per group, *p≤0.05, **p≤0.01, ***p≤0.001

Figure 3. Open-field after chronic treatment



Kruskal-Wallis ANOVA indicated significant stimulating effect of MDF on both distance travelled and rearings for the first time with no regard of prenatal treatment. All data are shown as means ±SEM, n=12 per group, *p≤0.05, **p≤0.01, ***p≤0.001.

Figure 4. Open-field after the challenge dose

Open-field after a challenge dose of MDF (PND 91)

The challenge MDF dose was the first to exert a significant stimulating effect on the control mice in both distance travelled and rearings. Specifically: in the distance travelled KW ANOVA showed a significant variability: H(3)=32.489, p<0.001 and multiple comparisons showed a significant differences between SAL-MDF and SAL-SAL mice (p=0.011) as well as MDF-MDF and MDF-SAL (p<0.001). Analogous results were observed in the rearing behaviour: H(3)=25.938, p<0.001, SAL-MDF vs. SAL-SAL (p=0.032) and MDF-MDF vs. MDF-SAL (p=0.001). No differences were present in the central zone behaviours.

Open-field behaviour development in time (PND 70-77-84-91)

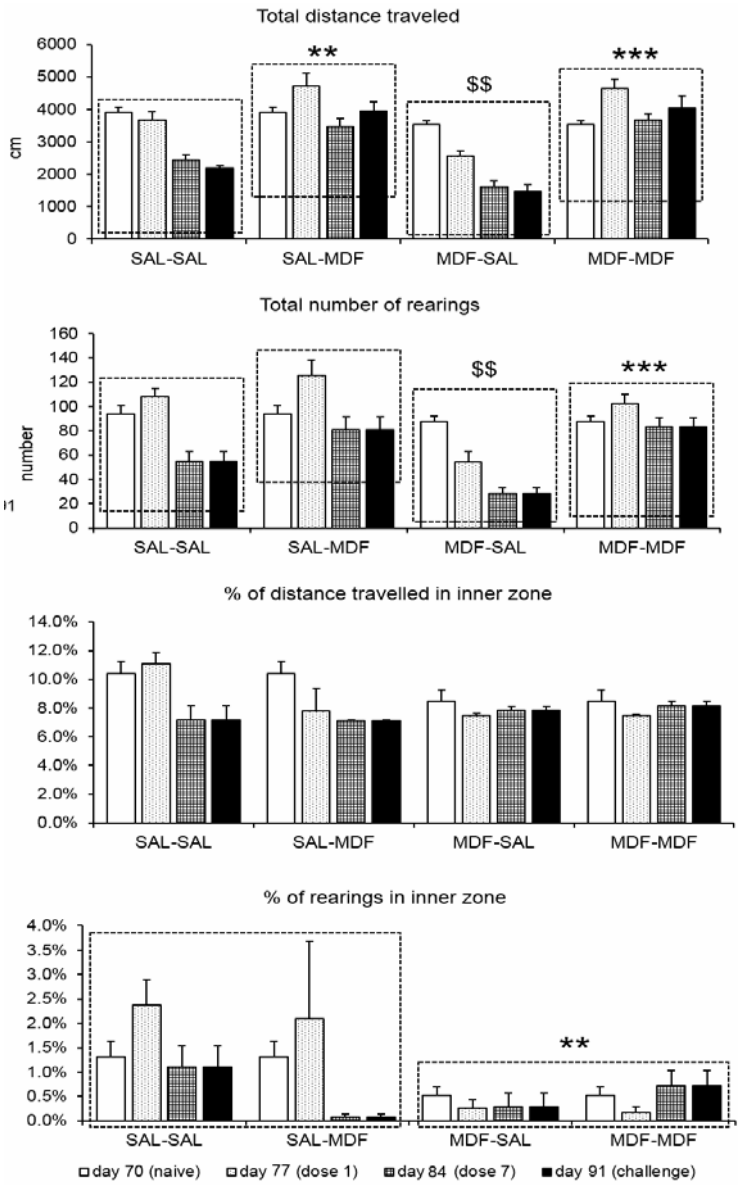
In order to estimate potential differences in the time-course of development and expression of behavioural sensitization induced by repeated intermittent MDF dosing, repeated measures ANOVA (two factors: prenatal and postnatal treatment, repetition: measurements) was employed. As shown in the Figure 5, the time-dependent MDF induced changes of distance travelled were significantly dissimilar from the SAL treated animals with both types of prenatal treatment but there was no difference between them. RM ANOVA revealed significant effects of both prenatal ($F_{(1,44)}$=5.78, p=0.021) and postnatal treatment ($F_{(1,44)}$=60.30, p<0.001) as well as prenatal*postnatal treatment interaction ($F_{(3,132)}$=5.41, p=0.025). Bonferroni post-hoc test for the prenatal*postnatal treatment interaction indicated specific differences between the groups: SAL-SAL vs. SAL-MDF, p=0.002 and MDF-SAL vs. MDF-MDF, p<0.001. Interestingly, the only difference attributable to prenatal MDF exposure was the locomotion supressing effect in the MDF-SAL animals compared to SAL-SAL, Bonferroni post-hoc test: p=0.010.

In the rearing behaviour analysis, the RM ANOVA also showed significant effects of prenatal ($F_{(1,44)}$=11.14, p=0.002), postnatal ($F_{(1,44)}$=26.27, p<0.001) and prenatal*postnatal treatment interaction ($F_{(3,132)}$=4.11, p=0.049). However, Bonferroni post-hoc test indicated a significant stimulating effect of MDF only in the MDF-SAL vs. MDF-MDF comparison (p<0.001) but not in the SAL-SAL vs. SAL-MDF mice. Another difference attributable to prenatal MDF exposure was the rearing supressing effect in the MDF-SAL animals compared to SAL-SAL, Bonferroni post-hoc test: p=0.003.

RM ANOVA did not indicate any significant differences in the proportion of distance travelled in the central zone. However, a significant effect of prenatal treatment ($F_{(1,44)}$=9.17, p=0.004) was found in the number of rearing episodes in the central part of the arena, confirmed by the Bonferroni post-hoc test, p=0.004.

Leucocyte phagocytic activity (PND 120)

For the analysis of the leucocyte chemiluminiscence data (Figure 6) a RM ANOVA indicated only a significant effect of prenatal treatment ($F_{(1,34)}$=5.57, p=0.024), Bonferroni post-test, p=0.023. This suggests that prenatal exposure to MDF may impair leucocyte phagocytic activity and consequently cause immune deficits.



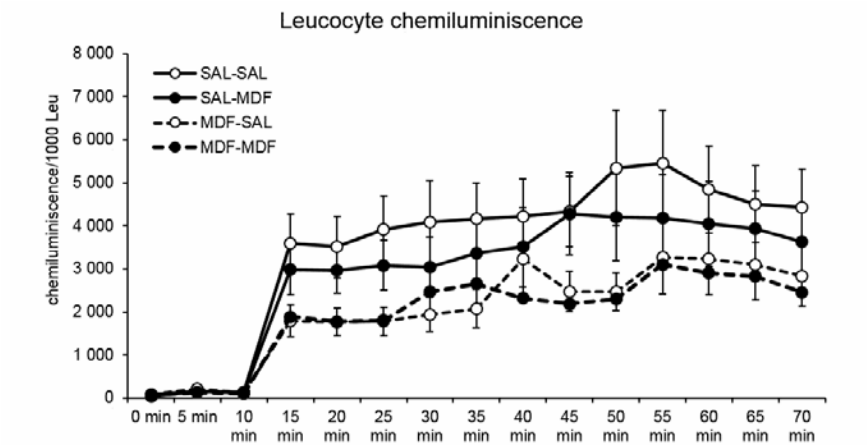
The graphs presented as means ±SEM show the data of each group in a timeline. Repeated measures ANOVA revealed significant differences in the groups (indicated by dotted boxes) marked as: *p≤0.05, **p≤0.01, ***p≤0.001 for the comparison of groups postnatally treated with vehicle and MDF with their prenatally exposed counterparts. $$p≤0.01 indicates difference between SAL-SAL and MDF-SAL animals. In the comparison of % of rearings in the centre of the arena, only prenatal treatment has a significant effect **p≤0.01

Figure 5. Time development of open-field behaviour over the course of the study

DISCUSSION

This study revealed significant detrimental effects of prenatal modafinil exposure leading to an altered behavioural profile, different reaction to postnatal modafinil treatment and impaired leucocyte activity. The main limitation for interpretation of these results is the absence of cross-fostering in this study, i.e. it is impossible to distinguish whether the observed effects appeared due to postnatal treatment or potentially impaired rearing behaviour of the modafinil treated mothers.

Specifically, we have observed a general hypolocomotion after prenatal modafinil exposure in adult mice evaluated by the open-field test. Furthermore, an increase in behavioural indicators of psychostimulant effects, i.e. distance travelled and incidence of rearing behaviour was higher in the modafinil prenatally exposed subjects. This effect was present after both acute and chronic treatment. However, it is important to note that all open-field measurements were performed under the immediate effect of the drug (30 minutes after administration). Therefore, we cannot rule out the possibility that even the reaction to the 8th dose of the drug exerted an acute effect. Also, the fact that the prenatally exposed animals were generally hypoactive probably contributed to the significant psychostimulatory effect observed in this group. Interestingly, the challenge dose led for the first time to significant increase in both horizontal and vertical locomotor behaviour in the control animals.



The graph shows means ±SEM in a 5-min timeline measurements, n=9-10 per group. Repeated measures ANOVA with Bonferroni post-hoc test revealed significant effect of the prenatal treatment indicating immune impairment in the MDF prenatally exposed mice (SAL-SAL + SAL-MDF vs. MDF-SAL + MDF-MDF, p=0.023)

Figure 6. Chemiluminiscence of phagocytes

The main behavioural effect of psychostimulants is increased locomotion (Wood et al. 2014). This was confirmed for modafinil as well (Simon et al. 1996) and is consistent with the previous finding that modafinil exerts expression but not development of behavioural sensitization (Paterson et al. 2010). Furthermore, modafinil-induced behavioural sensitization may be a prone to inter-individual variability, i.e. may develop only in some animals in the study (Soeiro Ada et al. 2012). However, when it comes to development of behavioural sensitization, classical psychostimulants are more effective. Modafinil at a dose comparable to our study (75 mg/kg) was reported to induce only a few signs of sensitization while cocaine showed robust effects (Shuman et al. 2012). This is in accordance with our data showing only a moderate tendency to develop behavioural sensitization after repeated modafinil treatment in control animals. Interestingly, prenatal exposure led to increased vulnerability to behavioural signs of psychostimulation induced by modafinil.

Psychostimulants tend to induce increased anxiety as described in caffeine (Nehlig et al. 1992), cocaine and amphetamines (Stanek 2006). In amphetamine-like drugs this effect is probably mediated via increased noradrenergic signalling which was shown to be involved in both elevated-plus maze and open-field readouts (Schmidt & Weinshenker 2014) and modafinil is known to have agonistic effect on noradrenergic receptors alpha-1 and inhibits noradrenaline transporter (Kim 2012). Therefore, it is possible to assume modafinil has a potential to be anxiogenic. In this study we used the open-field to assess the anxiety-like behaviour by calculating the proportion of locomotion in the central part of the arena as described earlier (Royce 1977, Choleris et al. 2001). We did not observe convincing evidence of anxiogenic effect of either prenatal or postnatal exposure to modafinil. There were some trends towards decreased rearing behaviour in the central part of the arena in the prenatally exposed mice but this profile was not shown in all measurements. This is in accordance with a study where modafinil dose range 32, 64 and 128 mg/kg did not alter elevated plus behaviour (Fernandes et al. 2015). In clinical studies, only modafinil overdose was shown to be anxiogenic (Spiller et al. 2009, Carstairs et al. 2010) while clinically relevant dose in healthy volunteers lead to improved mood and no anxiety (Taneja et al. 2007). This is in accordance with our preclinical studies showing the doses without stimulant effect on locomotor behaviour in both timid and aggressive agonistic behaviour in mice the drug exhibited anxiolytic-like (selective inhibition of defensive-escape behaviour) and antiaggressive-like (suppression of aggressive acts) effects (Machalova et al. 2010b).

Therefore, we might have not observed increased anxiety due to the selection of dose. The dose of 50 mg/kg is about 5-times higher than clinically relevant and in preclinical experimentation the doses range up to 600 mg/kg (Minzenberg & Carter 2008). In accordance with this explanation modafinil was shown to sporadically increase of noradrenaline, serotonin and dopamine in prefrontal cortex and striatum at an extreme dose of 600 mg/kg, while locomotor activity was significantly raised dose-dependently after 300 and 600 mg/kg (Rowley et al. 2014).

Mechanism of action of modafinil is largely unknown but it partially shares its mechanism of action with amphetamine-like substances, although there are important differences such as the modafinil-induced dopamine transported blockade (Kim 2012). Therefore, the developmental effects of amphetamines might be similar to those induced by modafinil. Analogously as in our study with modafinil, chronic prenatal administration of methamphetamine or MDMA in rats and a challenge dose of the same drug in adulthood did not alter anxiety-related behaviour in the elevated-plus test (Macuchova et al. 2016). However, positive results were reported as well (Navarro & Maldonado 2002, Slamberova et al. 2015). Furthermore, an open-field study revealed no effect of prenatal methamphetamine exposure in basal behavioural profile and decreased the reactivity

to the challenge dose of the drug in adulthood (Schutova et al. 2010), which does not apply to modafinil as shown in this study. Noteworthy, some tests might be less sensitive in capturing the differences in anxiety. Elevated-plus test can render different results but social interaction test and ultrasonic vocalizations seem to be sensitive in an analogous design (Armstrong et al. 2001, Clemens et al. 2004, Slamberova et al. 2015). On the other hand, open-field test was shown to have a limited validity for anxiety-related outcome (Prut & Belzung 2003). Therefore, our negative results on anxiety measures may be (besides dose choice) explained by the lack of sensitivity of this test. Furthermore, the issue of high methodological variability of behavioural and neurochemical approaches in developmental studies was raised recently and represents an important limitation of data interpretation (McDonnell-Dowling & Kelly 2015, 2016).

Importantly, the assessment of leucocyte phagocytic activity revealed an impairment of this capacity in the prenatally modafinil exposed subjects but no effect of the treatment in the adult age. The central nervous system is known to modulate the immune system and sleep disturbances are known to be correlated with systemic markers of inflammation (Lorton et al. 2006). Impaired leucocyte phagocytosis was already observed in the olfactory bulbectomy model of depression (Pistovcakova et al. 2008). This model is known for multiple behavioural and neurochemical disturbances (Song & Leonard 2005) but also disruption of the sleep architecture, specifically longer time of rapid eye movement time (Wang et al. 2012). However, acute modafinil did not produce important alterations in levels of melatonin, cortisol or growth hormone (Brun et al. 1998). Therefore, our data are in accordance showing no effect of postnatal modafinil treatment which moreover took place after a long wash-out period. Results from the pre-registration studies on reproductive and developmental toxicity revealed increased incidence in skeletal variations, embryo-foetal lethality at clinically relevant exposures and showed no teratogenic effect or impairment of growth or development of the offspring (EMA 2011). However, these studies do not include behavioural profile or immune changes. To our best knowledge this is the first report on such modafinil-induced alterations.

CONCLUSION

The most important finding of this study was the immunosuppressing effect, hypolocomotion and increased behavioural response to modafinil-induced psychostimulation caused by prenatal exposure to the same drug. We did not detect significantly altered anxiety-related behaviour in any group with respect to the pre- and postnatal treatments. This is the first evidence of developmental toxicity of modafinil which needs to be taken into account as a potential risk factor when modafinil is administered to women who may become pregnant.

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Contribution of individual authors:

Jana Ruda-Kucerova was responsible for the study design, co-developed the original idea, performed literature review, and organized the experimental work. She performed the statistical analysis, prepared data for presentation and wrote the first draft of the manuscript.

Petra Amchova contributed substantially to behavioural testing and collection of data. She cross-checked the materials and methods section of the manuscript.

Alena Machalova co-developed the original idea, performed literature review and contributed to the final version of the manuscript.

Jana Pistovcakova contributed substantially to behavioural testing and collection of data and contributed to the final version of the manuscript.

Alexandra Sulcova was involved in the design of the study and discussion of the data and contributed to the final version of the manuscript.

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Correspondence:

Jana Ruda-Kucerova, MD
Masaryk University, Faculty of Medicine, Department of Pharmacology
Kamenice 5, 625 00 Brno, Czech Republic
E-mail: jkucer@med.muni.cz