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Behavioral, hyperthermic and pharmacokinetic profile of *para*-methoxymethamphetamine (PMMA) in rats

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ABSTRACT

Despite poisoning with the ecstasy substitute *para*-methoxymethamphetamine (PMMA) being typically associated with severe hyperthermia and death, behavioral and toxicological data on this drug are missing. Herein we present the behavioral profile of PMMA, its hyperthermic potency and pharmacokinetic profile in rats. The effects of PMMA 5 and 20 mg/kg on locomotion, on prepulse inhibition (PPI) of acoustic startle reaction (ASR), on body temperature under isolated and crowded conditions and on the pharmacokinetics analyzed with gas chromatography mass spectrometry (GC-MS) were evaluated. PMMA increased overall locomotion with the higher dose showing a biphasic effect. PPI was decreased dose-dependently. The hyperthermic response was present only with PMMA 20 mg/kg and was accompanied by extensive perspiration under crowded conditions. Serum levels of PMMA peaked at approximately 30 min after both treatments; on the contrary the maximum brain concentrations of PMMA at 20 mg/kg peaked approximately 1 h after the administration, which was rather delayed compared to maximum after 5 mg/kg dose. These data indicate that PMMA has a similar behavioral profile to stimulants and hallucinogens and that the toxicity might be increased in a crowded environment. High doses of PMMA have a gradual penetration to the brain which might lead to the delayed peak concentrations and prolonged effects of the drug.

1. Introduction

The appearance of other psychoactive compounds than 3,4-methylendioxymethamphetamine (MDMA) including *para*-methoxyamphetamine (PMA) and *para*-methoxymethamphetamine (PMMA) in tablets sold as ecstasy was a standard finding over the previous decade e.g. (EMCDDA, 2003, 2004; Parrott, 2004; Bossong et al., 2010; Antia, 2009). PMA and PMMA as ecstasy substitutes reportedly mimic some of the psychological effects of MDMA and might also have hallucinogenic effects and compared to MDMA these compounds have delayed onset of effects (Shulgin and Shulgin, 1991; EMCDDA, 2003) (www.erowid.org). Both are much more toxic in humans than MDMA itself as is documented by many cases of severe intoxication and death of users all over the world e.g.(Kraner et al., 2001; Becker et al., 2003; Dams et al., 2003; Johansen et al., 2003; Lin et al., 2007; Lamberth et al., 2008). Clinical attributes of victims of PMA/PMMA poisoning seem to be similar to MDMA. Extreme hyperthermia was one of the most common symptoms. As with MDMA (Green et al., 1995, 2004) environmental factors like ambient temperature or crowded conditions and physical hyperactivity can increase the toxicity. Others include agitation, rhabdo- and cardiomyolysis, arrhythmias due to hyperkalemia, convulsions, coma and severe coagulopathy (Byard et al., 2002; Dams et al., 2003; Lin et al., 2007; Lamberth et al., 2008). An elevated health risk can also be supported by combining with other drugs especially mixtures with MDMA or other stimulants (Pilgrim et al., 2009). Long term consumption of these substituted amphetamines has also been associated with cardiovascular and cerebrovascular pathology (Kaye et al., 2009; Pilgrim et al., 2009). Although both of these compounds appear frequently as a mixture in tablets and are extremely dangerous there is little information on PMA and much less on PMMA throughout scientific literature.

PMMA appears in two isoforms with S(+)-enantiomer being the more active compound (Young et al., 1999). It is metabolized in the liver by CYP2D6 on cytochrome P450 mainly by *O*-demethylation to *para*-hydroxymethamphetamine (pholedrine) in rats (Bach et al., 1999; Staack et al., 2004; Staack and Maurer, 2005) and to a minor extent to PMA, and other metabolites (Staack et al., 2003; Rohanova and Balikova, 2009). Subcutaneous (s.c.) administration of PMMA 40 mg/kg to rats led to a peak in serum at 30 min and its metabolite PMA at 2 h. The maximum concentration of the drug in the brain was at 1 h after administration, while for the metabolite, PMA, it was at 2 h. PMMA tissue concentration exceeded plasma, the highest was achieved in the lungs (Rohanova and Balikova, 2009).

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Since PMA and PMMA are structurally closely related their mechanisms of action are presumably very close and seem to be similar to that of MDMA. These mainly involve the release of serotonin from synaptic terminals and to a lesser extent also dopamine (Hegadoren et al., 1995; Scorza et al., 1997; Daws et al., 2000; Gough et al., 2002; Callaghan et al., 2005; Romero et al., 2006). Contrary to MDMA, PMA and most likely also PMMA are strong inhibitors of monoaminoxidase (MAO) type A (Green and El Hait, 1980; EMCDDA, 2003; Freezer et al., 2005). A serotonin syndrome (Parrott, 2002; Ener et al., 2003) induced by concomitant inhibition of MAO and increased serotonin release make both drugs significantly dangerous.

Discrimination studies showed that PMMA belongs to a MDMA-like family of drugs, having less stimulant properties than even MDMA or PMA (Glennon et al., 1988, 1997; Young et al., 1999; Glennon et al., 2007). However, the effects of PMA on locomotor activity in other experiments demonstrate inconsistent results. Studies have shown inhibition of spontaneous activity, no change in locomotion and hyperlocomotion. Registering conditions like ambient temperature or housing of animals and doses of the drugs seems to play a significant role (Lopatka et al., 1976; Martin-Iverson et al., 1991; Hegadoren et al., 1995; Daws et al., 2000; Bustamante et al., 2004; Jaehne et al., 2005, 2007). Similar varying findings dependent on dosage and ambient temperature were found for changes in body temperature (Daws et al., 2000; Jaehne et al., 2005; Freezer et al., 2005; Jaehne et al., 2007). Since PMA is a serotonin releasing agent it is not surprising that it also induced typical serotonin mediated behavior including stereotypes, forepaw treading, penile erection, piloerection and proptosis of the eyes similarly to MDMA (Freezer et al., 2005).

In rats and mice the median subcutaneous lethal dose value (LD50) of PMMA was reported to be 80–100 mg/kg (Steele et al., 1992). Interestingly in mice the toxicity slightly increased when animals were housed aggregated in cages (Glennon et al., 1988). Comparable toxicity in rats was found for its active metabolite PMA (Nichols et al., 1975; Lopatka et al., 1976); however in another study PMA appeared much more toxic since 6.2 mg/kg PMA led to the death of animals within one week after administration (Smythies et al., 1967).

With respect to the high clinical relevance of PMMA poisoning we have decided to evaluate the behavioral profile of PMMA in rats. We have chosen relatively low doses (5 and 20 mg/kg) of the drug related to its LD50 values in rats and mice. Since it remains unclear whether PMMA has stimulant properties we have concentrated on locomotor effects in the open field. To evaluate its psychotropic and probably hallucinogenic potential (Geyer et al., 2001; Vollenweider and Geyer, 2001) sensorimotor gating in the prepulse inhibition test was analyzed. Further hyperthermic effects of PMMA were studied. As stated above, hyperthermia, a common symptom that was present in PMA/PMMA poisoning, is typically increased by the setting where these drugs are abused. Thus, to mimic such conditions we have evaluated the effects of PMMA on body temperature under isolated and crowded conditions. Finally, due to the fact that some phenylalkylamine derivatives have been shown to have a biphasic mode of action e.g. (Palenicek et al., 2008) and the penetration of PMMA to the brain has been shown to be gradual and slightly delayed related to blood (Rohanova and Balikova, 2009) we have monitored behavioral parameters in two time intervals after administration — during the expected onset of its action and during its expected highest brain levels. Blood samples and brains of animals were analyzed for PMMA content to correlate pharmacokinetic parameters with the observed effects.

2. Materials and methods

2.1. Animals

All experiments were carried out on adult male Wistar rats (SPF animals; Hannover breed Konárovice, Czech Republic) weighing 200–250 g. Animals were housed in pairs in a 12 h light/dark regime with temperatures 22 ± 2 °C and free access to a standard diet and water. The rats were given an acclimatization period of 7–10 days prior to the start of each experiment. During this period animals were weighed twice and handled four times during the cleaning of cages. 10 animals were used in each experimental group. Each subject was tested only once. To minimize the number of animals used, all rats from behavioral experiments were also used for determination of blood and brain levels of PMMA. All 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.

2.2. Drugs and chemicals

Para-methoxymethamphetamine hydrochloride (PMMA.HCl; synthesized at Charles University in Prague, Faculty of Pharmacy, Hradec Králové, CZR; 97–98% purity analyzed by gas chromatography-mass spectrometry (GC-MS)) was dissolved in a physiological saline. PMMA 5 and 20 mg/kg or a vehicle (0.9% NaCl) was administered subcutaneously in a volume of 2 ml/kg. Reference standard PMMA.HCl for toxicological analyses and internal standard methamphetamine-D5.HCl for calibration and method validation were supplied by Sigma Aldrich Inc. and Lipomed Arlesheim AG. The reagents used for these purposes were of general analytical grade purity.

2.3. Toxicological analyses

2.3.1. Sample preparation for GC-MS

2.3.1.1. Serum. 0.5 ml serum was mixed with 0.5 ml 0.1 M phosphate buffer pH 6 and 400 ng deuterated methamphetamine (internal standard, IS). Analytes were isolated using SPEC-DAU discs. The eluates obtained were evaporated to dryness at 40 °C and then derivatized with 200 µl of an acetic acid anhydride/pyridine (10/1, v/v) mixture at 60 °C for 30 min, then evaporated again, transferred into 100 µl ethylacetate and subsequently 1 µl was injected for GC-MS analysis.

2.3.1.2. Brain tissue. 1 g whole brain tissue with 400 ng deuterated methamphetamine (IS) were homogenized with 4 ml methanol and subsequently submitted to sonification for 15 min. After centrifugation (5 min at 2400 × g), 2 ml of supernatant was separated, evaporated to dryness and reconstituted in 100 µl methanol and 1 ml 0.1 M phosphate pH 6 for isolation on SPEC-DAU discs and acetylation as described for the serum.

2.3.2. GC-MS conditions

A HP 6890-5973 (Agilent, Waldbronn, Germany) instrument was used equipped with an autosampler, splitless injector, and HP5-MS 30 m × 0.25 mm × 0.25 µm capillary. The carrier gas was helium at 1 ml/min constant flow. The oven temperature was programmed starting from 85 °C, held for 2 min, then 30 °C/min up to 150 °C, then 15 °C/min up to 250 °C and then held for 10 min. The injector and transfer line were held at 250 °C. Ionization was carried out by electron impact (EI) at 70 eV. SIM mode was used to assay PMMA in its acetylated form with monitoring ions of *m/z* 221, **148**, 121, 100, 58 and for the deuterated methamphetamine (internal standard) *m/z* 196, 119, **104**, 92, 62. The underlined ions were used for quantification.

2.3.3. Determination of PMMA levels in serum and brain samples

For this kinetic study a single bolus dose of PMMA 5 or 20 mg/kg was injected and subsequently 10 animals per group were sacrificed after 30, 60, 120, 240 and 480 min. To minimize the number of animals used, rats tested in behavioral experiments described below

were involved in the kinetic study. For control purposes physiological saline solution was injected to animals as well. Separated sera and whole brains were kept at −20 °C until the toxicological analysis. A GC-MS toxicological method was developed and validated according to international standards, e.g. (Lindner and Wainer, 1998; Penders and Verstraete, 2006; Peters et al., 2007) to determine the PMMA concentrations in rat serum and brain tissue samples under the operating conditions described above. Blank rat sera and brain tissue spiked with PMMA and the internal standard were used in the method validation process and for calibration purposes. The calibration curves were based on seven points in duplicates and were constructed using linear regression analysis based on the ratio of PMMA peak area related to the internal standard with regression coefficients better than 0.992, as it has previously been described in detail (Rohanova and Balikova, 2009).

2.4. Behavioral experiments

All behavioral experiments were conducted under standard temperatures and humidity conditions as in the animal facility: humidity ranging from 30 to 70% and temperature setting 22 ± 2 °C. All behavioral testing was performed in two separate temporal constellations with the beginning of testing at 15 and 60 min after PMMA administration. Separate groups of animals were used for each administration time.

2.4.1. Open field (Ethovision)

Locomotor activity (trajectory length) and its spatial characteristic (thigmotaxis and time spent in the center of the arena) in a novel environment were registered and analyzed by an automatic video tracking system for recording behavioral activities (EthoVision Color Pro v. 3.1.1, Noldus, Netherlands). A square black plastic box arena (68 × 68 × 30 cm) was situated in a sound-proof and evenly-lit room. We use low levels of light intensity to keep the room sober but sufficient enough for the camera to detect the animal in the arena; the light is equally spread within the experimental room from the spot sources by reflecting it off the white walls. Each rat was placed into the center of the arena 15 or 60 min after drug administration (PMMA 5 and 20 mg/kg or saline) and locomotor activity was registered for 30 min. The EthoVision program was also used to calculate locomotor activity in 5 min time intervals. To evaluate the spatial characteristics of the movement in the open field (thigmotaxis and time spent in the center of the arena) the arena was virtually divided by the EthoVision program into 5 × 5 identical square zones with 16 being located on the periphery and 9 centrally. The total number of appearances of the animal in each zone (frequency; f) was computed by the program and used in the calculation of thigmotaxis (i) in the following manner: $i = f_{\text{peripheral zones}} / f_{\text{all zones}}$. Thus, the thigmotaxis is a relative number which varies from 0 to 1 and indicates the probability of appearance in any of the peripheral zones within the arena. As a complementary measure, time spent in the center of the arena (T_{center}) was analyzed, equaling the summation of time spent in the 9 central zones (Σt_{1-9}) (Palenicek et al., 2005b, 2008, 2010). These measures can reflect alterations in exploratory behavior, can be associated with anxiety and can also describe stereotyped behavior (Lát, 1973; Wishaw et al., 1999; Palenicek et al., 2005a, 2007b, 2008, 2010).

2.4.2. Prepulse inhibition (PPI) of acoustic startle (ASR)

All of the rats were habituated to the testing apparatus in a short session (a 5 min acclimatization period plus five single pulses 2 days before the experiment).

All testing was performed in two startle chambers (SR-LAB, San Diego Instruments, California, USA) which consist of a sound-proof, evenly-lit enclosure with a Plexiglas stabilimeter with a 8.7 cm inner diameter. A piezoelectric accelerometer detected all peak and average amplitudes of the startle response. These were digitized and stored on a computer hard drive. A dynamic calibration system was used to ensure comparable stabilimeter sensitivity across the test chambers. Sound levels were measured using a RadioShack sound level meter. A high frequency loudspeaker mounted 24 cm above the Plexiglas cylinder inside the chamber produced both a background noise of 75 dB and all acoustic stimuli. The experimental design was adopted from previous studies (Bubenikova et al., 2005; Palenicek et al., 2008, 2010). After the acclimatization period (5 min) the test began with the first session consisting of four initial startle stimuli (125 dB). It was followed by the second session which consisted of four different trial types presented in a pseudo-random order: (1) single pulse: 125 dB broadband burst, 20 ms duration; (2) prepulse–pulse: prepulse 13 dB above the background noise, 20 ms duration, presented 100 ms before the onset of the 125 dB pulse alone; (3) prepulse alone: 13 dB above the background noise, 20 ms duration; (4) no stimulus. Five presentations of each trial type were given with a floating interstimulus interval of about 30 s. The PPI was expressed as a percentage of PPI [100 − (mean response for the prepulse–pulse trials / startle response for the single pulse trials) × 100]. The four single pulse trials at the beginning of the first session were not included in the calculation of the PPI values. Animals with an average startle value (the amplitude of the response) lower than 10 mV were excluded from the calculation of the PPI and were marked as non-responders.

2.4.3. Temperature measurements

Rats were divided into two groups: those that were housed separately (isolated condition) and those that were housed 5 animals in a cage (crowded condition); we used 10 animals per each treatment (PMMA 5 or 20 mg/kg and saline) and housing condition (isolated vs. crowded). In total we used 60 animals. All measurements were taken during the light phase of the cycle under the constant conditions in the animal facility as stated above. Rectal temperature was measured by a digital thermometer starting at 7:00. The first three measurements (first 3 h including the measurement at 7:00) were taken under drug-free conditions every hour until 9:00. PMMA (5 or 20 mg/kg) or saline was administered at 9:00, and the temperature was recorded in 0.5 h intervals for 2 h. After this, temperature was measured every hour until 17:00. During the measurement rats were shortly immobilized in a Plexiglas tube, the temperature measurement lasted 10 s. In addition to temperature measurements we also observed animal perspiration in the same intervals. To semi-quantify the magnitude of perspiration we used symbols: + slight perspiration (small parts, 10–20%, of the body surface wet), ++ intermediate perspiration (approximately 20–50% of the body surface wet), and +++ excessive perspiration (more than 50% of the body surface wet).

2.5. Statistical analysis

All statistical analyses were conducted using SigmaStat v. 3.0 or Statistica v. 7.0 software. The differences between groups with $p < 0.05$ and lower were considered significant.

Statistical analysis of total locomotion and PPI ASR was conducted by two-way analysis of variance (ANOVA) with PMMA treatment and time of administration as factors. To evaluate the effect of PMMA in 5 min time intervals a three-way repeated measures (RM) ANOVA with treatment and time of administration as between-subjects factors and time interval as repeated measures factor was used. In the temperature analysis again a three-way RM ANOVA was used with treatment and housing condition as the between-subjects factors and time interval as a repeated measures factor. All ANOVAs were followed by Bonferroni post hoc tests where appropriate.

3. Results

3.1. Pharmacokinetics of PMMA in serum and brain tissue (Fig. 1)

The mean concentration values (n = 10) are presented in the Fig. 1 separately for each dose, where the time profile of PMMA both in serum and the brain are apparent with a clear time delay of the concentration maxima attained in brain related to serum. The PMMA brain levels significantly exceeded those in serum which indicates the high degree of drug incorporation into the brain. The temporal profile of PMMA in the brain may be associated with the time course of some of its psychotropic effects.

3.2. Locomotion

3.2.1. Total locomotion (Fig. 2)

Two-way ANOVA of total locomotion (trajectory length within 30 min) confirmed a significant effect of treatment ($F_{(2,54)} = 13.781$, $p<0.001$) but not of time of administration. There was also no interaction between factors indicating no specific differences between the two experiments which differed by the time of administration of the substance. PMMA induced hyperlocomotion in both experiments as shown by post hoc analysis. Both doses of PMMA increased locomotion compared to the control group ($p<0.05$–$p<0.001$) at 60 min after administration, while in the experiment when PMMA was administered at 15 min before testing only PMMA 5 mg/kg reached significance ($p<0.01$), PMMA 20 mg/kg showed a trend to increase locomotion ($p = 0.056$) (Fig. 2A/B).

3.2.2. Total locomotion in 5 min intervals (Fig. 3)

Three-way RM ANOVA showed an effect of treatment ($F_{(2,270)} = 13.782$, $p<0.001$), but not of time of administration nor was there an interaction between treatment and time of administration. In addition, it revealed the effect of time interval ($F_{(5,270)} = 58.24$, $p<0.001$) and all other interactions ($F_{(10,270)} = 30.98$, $p<0.001$ for time interval × treatment, $F_{(5,270)} = 6.14$, $p<0.001$ for time interval × time of administration and $F_{(10,270)} = 5.73$, $p<0.001$ for time interval × treatment × time of administration) (Fig. 3).

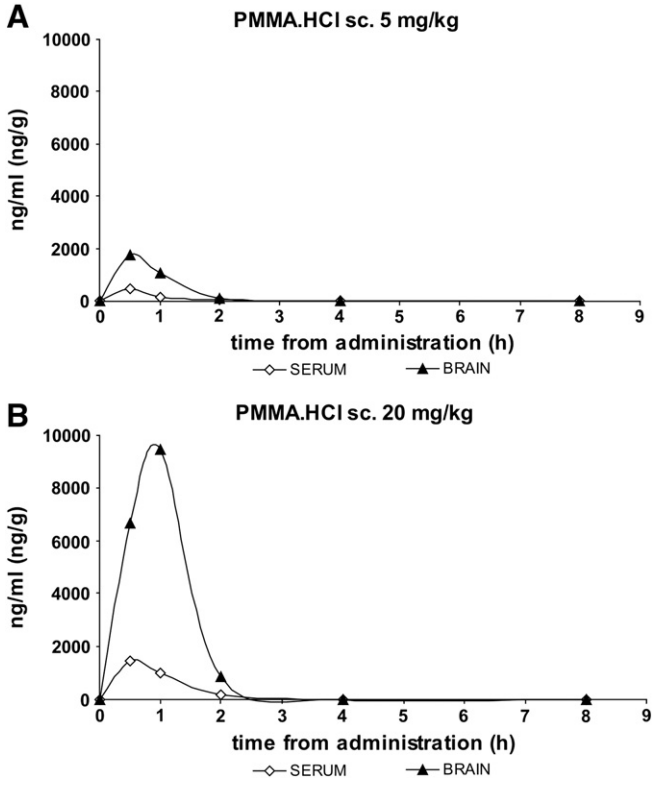

Fig. 1. Temporal profile of serum and brain levels of PMMA. Blood serum and brain tissue levels of PMMA.HCl after 5 mg/kg s.c. (A) 20 mg/kg s.c. (B).

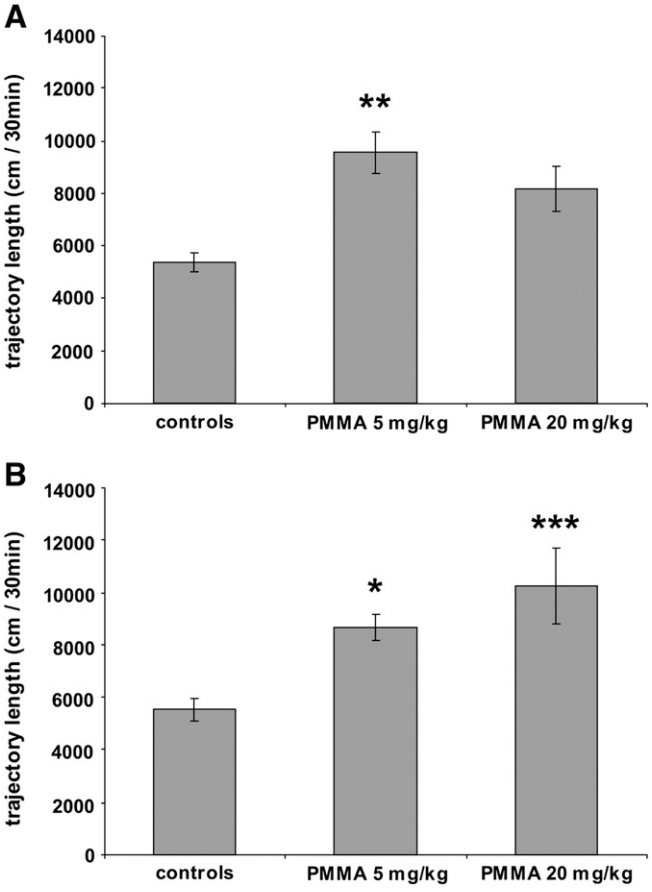

Fig. 2. Effect of PMMA on total locomotion 15 min after administration (A) and 60 min after administration (B). Both doses of PMMA increased the total locomotion in both temporal arrangements, there were no significant differences between the two experiments. *, **, ***Indicates $p<0.05$–$p<0.001$ from the respective vehicle (control) group (two-way ANOVA with post hoc Bonferroni tests).

The post hoc analysis of time effects (comparing 5 min intervals within the 30 min of observation) pointed that:

A) In control animals administered with the vehicle 15 or 60 min before the measurement the locomotion constantly decreased during the measurement; almost all intervals differed from the first and second intervals ($p<0.001$).
B) Animals treated with PMMA 5 mg/kg 15 min before measurement displayed a U shape of locomotor effects. Initially the locomotion slightly decreased during the second–fourth interval in a comparable manner as in the control animals. The trajectory in the first interval was significantly longer than in any other ($p<0.01$–$p<0.001$), similarly it was longer in the second interval when compared to the third and fourth intervals ($p<0.01$). However the locomotion started to slightly increase from the fifth interval and the locomotion in the last interval was longer compared to the third and fourth intervals ($p<0.01$). When PMMA 5 mg/kg was administered 60 min before measurements, the locomotion constantly decreased in a similar manner as in the control animals; all subsequent intervals differed from the first interval ($p<0.001$), and equally for the second interval ($p<0.05$–0.001).

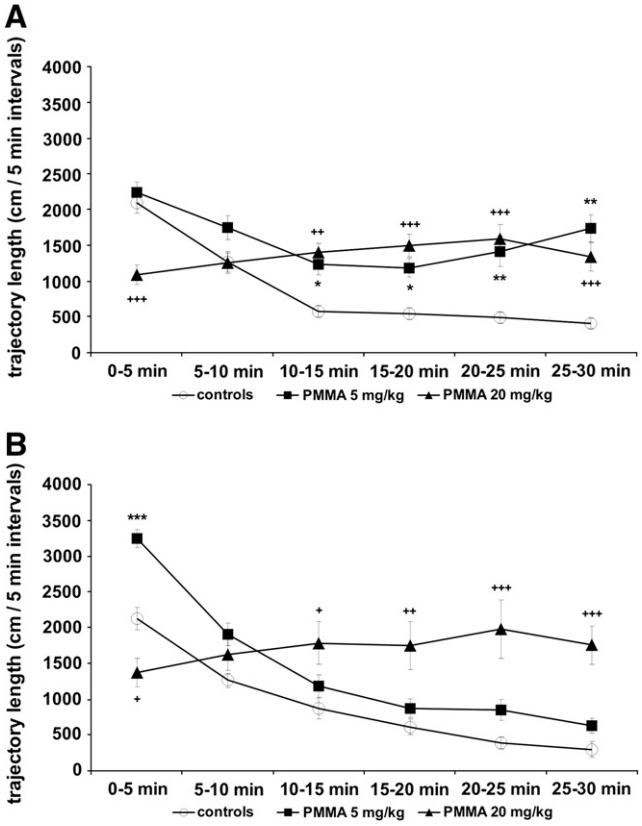

Fig. 3. Locomotor effects of PMMA in 5-min intervals at 15 min after administration (A) and 60 min after administration (B). (A) PMMA 5 mg/kg induced U shape of locomotor changes (initially a decrease followed by an increase); during the second half of the measurement the trajectory was significantly longer compared to the controls. PMMA 20 mg/kg after initial hypolocomotion led to hyperlocomotion. (B) PMMA 5 mg/kg when compared to the controls induced an increase in locomotion during the first two intervals, no change was observed in subsequent intervals, PMMA 20 mg/kg after initial hypolocomotion (compared to the controls) led to an increase in locomotion in the second half of the measurement (compared to the controls as well as to PMMA 20 mg/kg in the first interval). *, **, ***Indicates $p<0.05$–$p<0.001$ from the vehicle (control) group for PMMA 5 mg/kg. $^{++}$, $^{+++}$Indicates $p<0.01$–$p<0.001$ from the vehicle (control) group for PMMA 20 mg/kg (three-way RM ANOVA with Bonferroni post hoc tests).

C) After PMMA 20 mg/kg administered 15 min before measurement locomotion slightly increased at fourth ($p=0.08$) and fifth ($p<0.05$) interval when compared to the first interval; no other differences were observed. When PMMA 20 mg/kg was administered 60 min before the measurement again in the fifth interval the locomotion was slightly longer than in the first interval ($p=0.05$).

The treatment effects in each 5 min interval within the registered 30 min period showed:

A) In animals administered 15 min before the experiment with PMMA 5 mg/kg the trajectory length was significantly longer than in vehicle treated animals in all intervals from 10 to 15 min after the beginning of the session ($p<0.01$–$p<0.001$). On the contrary, after administration PMMA 20 mg/kg treated animals initially had shorter trajectory than control animals (during the 0–5 min interval, $p<0.001$), subsequently starting from the third interval the effect was opposite ($p<0.001$).
B) At 60 min after administration PMMA 5 mg/kg treated animals showed significantly longer trajectory than controls during the first interval ($p<0.001$) and almost during the second interval ($p=0.081$), subsequently in all other intervals the locomotion was comparable to the controls. PMMA 20 mg/kg had a similar mode of action as when administered 15 min before the measurement. Initially animals displayed shorter trajectory than controls during the first interval ($p<0.001$), subsequently from the third interval the distance moved was longer than in the control animals ($p<0.01$–$p<0.001$).

3.3. Thigmotaxis (i) and time spent in the center of the arena (T_{center}) (Fig. 4A/B/C)

Two-way ANOVA of thigmotaxis confirmed a significant effect of treatment ($F_{(2,54)}=32.561$, $p<0.001$) but not of time of administration, nor their interaction. Subsequent post hoc tests showed that PMMA 20 mg/kg significantly decreased thigmotaxis ($p<0.001$ in both experiments) (Fig. 4A/B/C).

Two-way ANOVA for time spent in the center again showed an effect of treatment ($F_{(2,54)}=50.237$, $p<0.001$) but not of time of administration, nor their interaction. Post hoc tests showed that administration of PMMA at 15 min before measurement increased dose dependently time spent in the center ($p<0.05$–$p<0.001$). Administration of PMMA at 60 min before measurement significantly increased time spent in the center only in PMMA 20 mg/k ($p<0.001$) while the same direction of changes in PMMA 5 mg/kg is also obvious. Further a focus on trajectory revealed that animals treated with PMMA 20 mg/kg showed stereotyped circulating in the central parts of the arena. Proportionally to the whole length of the measurement $\boldsymbol{T}_{center}$ increased to 25.1% and 56.6% after PMMA 5 and 20 mg/kg compared to 4.6% in vehicle treated animals when drugs were administered 15 min prior testing and identically to 17.3% and 48.4% compared to 5.6% in the case when drugs were administered 60 min prior to testing.

3.4. Acoustic startle reaction (ASR) (Table 1) and prepulse inhibition (PPI) of acoustic startle reaction (Fig. 5A/B)

The number of animals excluded from the analysis due to low startle reaction (average value lower than 10) did not significantly differ among all treatment groups (maximum 1 animal per group) (Fig. 5A/B).

Two-way ANOVA of ASR showed a significant effect of treatment ($F_{(2,53)}=4.283$, $p<0.05$) but not of time of administration nor for interaction between factors. Post hoc tests did not find any difference between PMMA treatments and control group, yet a trend of PMMA 5 mg/kg to decrease ASR is obvious (see Table 1).

Two-way ANOVA for the effect on PPI showed a significant effect of treatment ($F_{(2,53)}=15.909$, $p<0.001$), but not of time of administration and none for interaction. Post hoc analysis revealed that both doses of PMMA significantly decreased PPI from their respective control group ($p<0.05$–$p<0.001$); the only insignificant observation was for PMMA 5 mg/kg administered 15 min before testing where the post hoc test showed only a trend to disrupt PPI ($p=0.065$).

3.5. The effects of PMMA on body temperature (Fig. 6A/B)

Three-way RM ANOVA showed an effect of treatment ($F_{(2,648)}=30.2$, $p<0.001$), housing condition ($F_{(1,648)}=5.3$, $p<0.05$) and time ($F_{(12,648)}=192.4$, $p<0.001$). There was no interaction between housing conditions and treatment but there were interactions between time and housing conditions ($F_{(12,648)}=7.2$, $p<0.001$) indicating a temporal difference between the two housing conditions. This was obvious mainly in PMMA 20 mg/kg treated animals where the increase in temperature was faster in animals housed in groups than in those housed separately and that the temperature remained elevated until the end of the measurement under the crowded condition. Other interactions were also present: time × treatment ($F_{(24,648)}=9.1$, $p<0.001$) and even triple interaction time × treatment × housing condition ($F_{(24,648)}=2.5$, $p<0.001$).

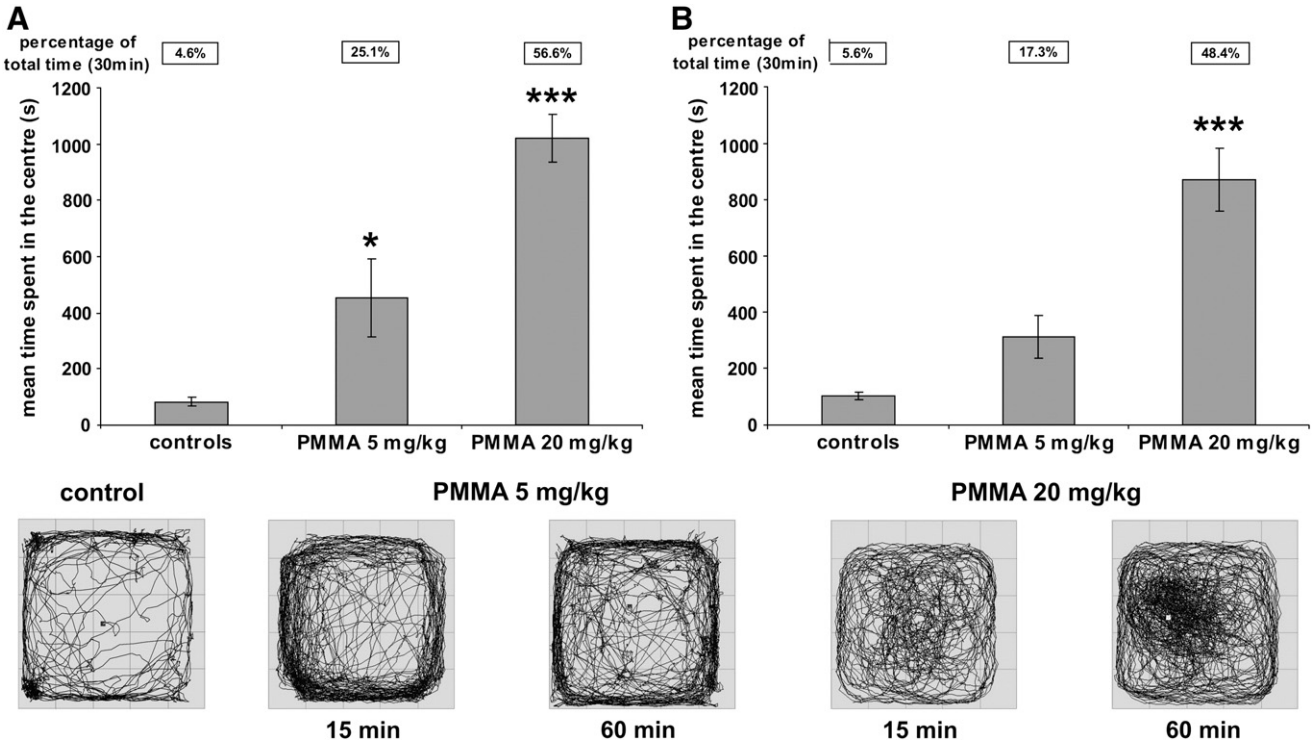

Fig. 4. Effect of PMMA on time spent in the center of the arena 15 min after administration (A) and 60 min after administration (B). Examples of characteristic trajectories (C). PMMA increased time spent in the center in both experiments; there were no significant differences between the 15 and 60 min administration scheme. Each trajectory image represents characteristic trajectory of one animal (treated with either saline, PMMA 5 or 20 mg/kg) within the whole length of measurement (30 min). *, ***Indicates $p<0.05$ and $p<0.001$ from the vehicle (control) group (two-way ANOVA with post hoc Bonferroni tests).

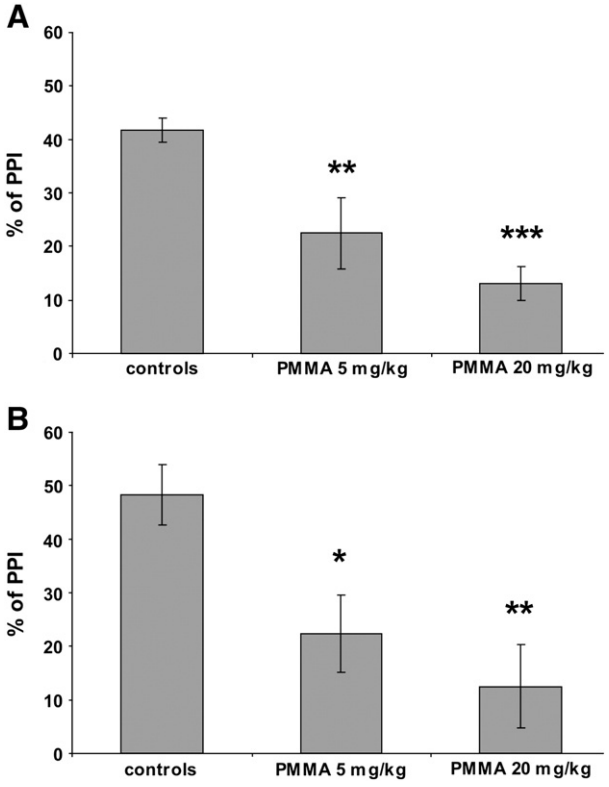

Fig. 5. Effect of PMMA on PPI ASR 15 min after administration (A) and 60 min after administration (B). PMMA disrupted PPI in both experiments; there were no significant differences between the 15 and 60 min administration scheme. *, **, ***Indicates $p<0.05$–$p<0.001$ from the vehicle (control) group (two-way ANOVA with Bonferroni post hoc tests).

3.5.1. Temperature changes in PMMA treated animals when compared to animals treated with the vehicle

Subsequent post hoc analyses comparing the effect of treatment to the control animals showed that in animals housed separately the temperature after PMMA 20 mg/kg started to increase at 1 h after the drug administration with a maximal peak at 1.5 h and remained significantly higher for another 4 h after the drug administration. On the contrary, PMMA 5 mg/kg slightly decreased the temperature at 30 min after administration with no other changes during all observations. None of the doses induced perspiration under these conditions. In animals housed five in a cage the temperature after PMMA 20 mg/kg started to increase at 30 min with the maximal peak at 1 h after administration and remained significantly higher for 6 h after drug administration until the end of the analysis. Animals started to perspire at 30 min after administration, during the next two observations at 1 and 1.5 h after administration they were excessively perspiring with a subsequent decline in perspiration up to 3 h after the drug administration. There was no major change in temperature or in perspiration in animals treated with PMMA 5 mg/kg. Results of post hoc analyses showing differences from control animals are shown in Fig. 6A/B.

Table 1
The effect of PMMA on acoustic startle response (ASR). This table shows ASR magnitude; there were no statistical differences in ASR, however a decrease in ASR after 5 mg/kg was obvious (two-way ANOVA with post hoc Bonferroni tests).

Time of administration	Mean average ASR amplitude in mV (±SEM)		
	Vehicle	PMMA 5 mg/kg	PMMA 20 mg/kg
15 min	103.6 (±22.5)	71.7 (±7.2)	106.3 (±7.4)
60 min	124.4 (±13.5)	84.9 (±8.9)	111.2 (±13)

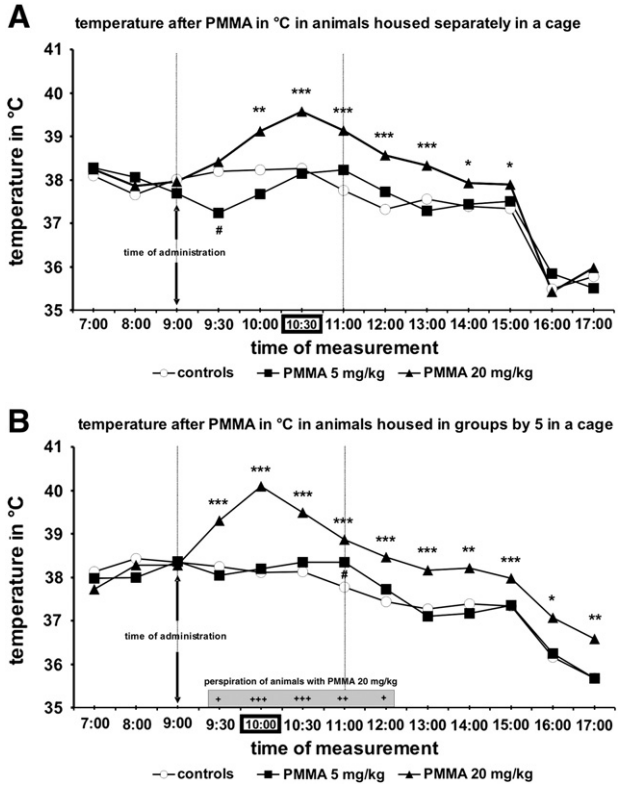

Fig. 6. The effects of PMMA on body temperature in animals housed separately (A) and in groups (B). PMMA 20 mg/kg significantly increased temperatures in both experiments; the temperature increase persisted longer in animals housed in groups. In animals housed in groups excessive perspiration was also observed. Only minor effects were observed in PMMA 5 mg/kg treated animals. Perspiration of animals is marked with +, black rectangle on the timeline indicates the time of the highest mean temperature for PMMA 20 mg/kg. The area between the vertical dotted lines indicates that temperature was measured in 0.5 h intervals, elsewhere measurements were done in 1 h intervals.#Indicates $p<0.05$ from the vehicle (control) group for PMMA 5 mg/kg. *, **, ***Indicates $p<0.05$–$p<0.001$ from the vehicle (control) group for PMMA 20 mg/kg (three-way RM ANOVA with post hoc Bonferroni tests).

3.5.2. The time characteristics of temperature changes in animals housed separately

In control animals the temperature remained almost constant from 7:00 to 10:30, when it decreased continuously until 17:00. In the last two measurements the temperature was significantly lower than during the rest of the day ($p<0.001$ for all comparisons). In PMMA 5 mg/kg treated animals there was an initial slight decrease in temperature after the PMMA administration at 9:30 ($p<0.001$ compared to first measurement) followed by an increase at 10:30 to temperatures comparable to the beginning of the measurement. Subsequently, from 10:30 the curve almost copied the control animals and the temperature constantly decreased until 17:00, again the last two measurements showed significantly lower temperatures than during the rest of the day ($p<0.001$ for all comparisons). PMMA 20 mg/kg induced an increase in temperature from 10:00 (1 h after administration) which continued until 11:00 ($p<0.001$ compared to first three measurements). Subsequently, the temperature started to decrease and from 15:00 it copied the temperature of control animals. The decrease of temperature in the last two measurements was significant compared to the others ($p<0.001$ for all comparisons).

3.5.3. The time characteristics of temperature changes in animals housed separately

Control animals had a relatively constant temperature from 7:00 to 10:30, later the temperature declined continuously until 17:00 ($p<0.05$–0.001 compared to measurement at 10:30 and to the second and third measurements). Again the last two measurements displayed a significant drop in temperature compared to the others ($p<0.001$ for all comparisons). In PMMA 5 mg/kg treated animals the temperature curve almost copied the control one. With a 1 h delay compared to the controls the temperature continuously decreased from 12:00 to 17:00 ($p<0.05$–0.001 compared to measurements within 9:00–11:00), with the most prominent decrease in temperature being observed again at 16:00 and 17:00 ($p<0.001$ for all comparisons). PMMA 20 mg/kg increased the temperature at 30 min after administration of the compound (9:30); when compared to the first three intervals the temperature remained significantly high until 11:00 ($p<0.001$). Subsequently, the curve continued to copy the control curve (however the temperature was approximately 0.5 °C higher than in the controls) with a characteristic decline in temperature between 16:00 and 17:00 ($p<0.05$–0.001 compared to the measurement at 10:30 and to the first three measurements). The afternoon decline in temperature was again significant when compared to all other measurements ($p<0.001$ for all comparisons).

4. Discussion

The main findings revealed overall stimulatory effects of PMMA on locomotion, disruption of sensorimotor gating and hyperthermia accompanied with extensive perspiration in crowded conditions. The time course of locomotor changes of the lower PMMA dose was similar to that of the control animals, however an upper shift was evident; the higher PMMA dose initially induced a decrease followed by an increase of locomotion. While PPI was disrupted by both PMMA doses only a decreasing trend of ASR with PMMA 5 mg/kg was present. On the contrary to the high PMMA dose which induced hyperthermia, the lower dose had no constant effect on body temperature except for an initial slight decrease under isolated conditions.

PMMA concentrations in serum achieved a maximum after 30 min for both doses with a rapid decline. Although some traces could be detected for the higher dose after 4 h, none could be detected after 8 h by our method (limit of detection 10 ng/ml). Incorporation into the brain was fast; nevertheless the maximum concentration after the higher dose was achieved rather later than after the lower dose (approximately 60 min related to 30 min). In our previous work using very high PMMA doses (40 mg/kg) a delay in peak brain concentrations and longer persistence in the brain were also observed (Rohanova and Balikova, 2009). This may be explained by sequestration of the drug into other compartments, mainly lung tissue, where the higher doses of PMMA can accumulate and be gradually released (Rohanova and Balikova, 2009). Then the duration of the psychotropic effect is associated with the drug dose applied. The drug dose seems to have differing impacts for behavioral observation in our study.

PMMA induced hyperlocomotion independently of time of administration. Locomotor effects of PMMA 5 mg/kg were more pronounced during the onset of its action while PMMA 20 mg/kg had more stimulatory effects 1 h after administration. Focus on the locomotor effects revealed that PMMA 20 mg/kg produced almost constant, slightly increasing locomotion over the testing session. Initially there was decreased locomotion even compared to control animals, an effect that was not observed with PMMA 5 mg/kg. PMMA 20 mg/kg also significantly increased the time spent in the center of the arena as well as decreased thigmotaxis. More detailed examination of the trajectory revealed that animals were stereotypically circulating in the central parts after a high PMMA dose (Fig. 3C).

To our knowledge there is only one previous study with PMMA in rodents. Contradictory to our results, the authors in this study described that PMMA 8 mg/kg decreased spontaneous motor activity, rearing and grooming (Bustamante et al., 2004). However, the same work also states that the same dose of its congener PMA did not have any effect on these behavioral measures. Even though data on PMMA are missing throughout the literature, the comparable data from most

related compounds PMA, MDMA and methamphetamine are available. In rodents PMA in doses ranging from 0.8 to 10 mg/kg brought inconsistent results characterized by no effect (Martin-Iverson et al., 1991; Hegadoren et al., 1995; Daws et al., 2000; Jaehne et al., 2007), reduction (Lopatka et al., 1976) or an increase of animal activity and/or locomotion (Daws et al., 2000; Freezer et al., 2005). If present, stimulatory effects of PMA on behavior were comparable to equivalent doses of MDMA (Freezer et al., 2005; Palenicek et al., 2005b, 2007b) and high ambient temperature potentiated these effects (Jaehne et al., 2005). Regarding our results PMMA also had stimulant potency comparable to MDMA (McCreary et al., 1999; Bankson and Cunningham, 2002; Bubar et al., 2004; Freezer et al., 2005; Palenicek et al., 2005b, 2007b) but it seemed to be weaker than methamphetamine (Fukushima et al., 2007; Hall et al., 2008; Schutova et al., 2009). Interestingly the biphasic locomotor effect of PMMA 20 mg/kg resembles findings with hallucinogenic drugs like mescaline, DMT (N,N-dimethyltryptamine), psilocin, DOM (2,5-dimethoxy-4-methylamphetamine), or DOET (2,5-dimethoxy-4-ethylamphetamine) (Geyer et al., 1979; Palenicek et al., 2008). The initial hypolocomotion observed with the higher dose might also be a result of ataxia as was described for mescaline, LSD and other hallucinogens (Sykes, 1986; Krebs-Thomson and Geyer, 1996; Krebs-Thomson et al., 1998b, 2006; Palenicek et al., 2008, 2010). The occurrence of stereotyped behavior is also comparable to MDMA, stimulants and hallucinogenic drugs (Gold et al., 1989; Paulus and Geyer, 1992; Abekawa et al., 1994; Ball et al., 2003; Gentry et al., 2004; Freezer et al., 2005; Palenicek et al., 2005b, 2007b, 2008). A plausible explanation for the difference between the time course of the effects of low and high dose is a loss of selectivity of the drug with its higher dose, or more robust effect on serotonergic than dopaminergic systems. In line with this, more selective serotonin releasers like fenfluramine or meta-chlorophenylpiperazine (mCPP) typically induce hypolocomotor effects (Lindquist and Gotestam, 1977; Martin et al., 2002; Khaliq et al., 2008), while dopaminergic agents typically exert hyperlocomotion (Fukushima et al., 2007; Hall et al., 2008; Schutova et al., 2009). In conclusion, the above mentioned facts on locomotor changes and occurrence of stereotyped locomotor behavior indicate that PMMA displays mixed stimulatory and hallucinogen-like properties.

PPI was disrupted in a dose dependent manner in both temporal arrangements. Interestingly, PMMA 5 mg had lower concentrations in the brain 1 h after administration and PPI remained disrupted. To our knowledge there are no previous studies with either PMA or PMMA on PPI. On the other hand, MDMA and hallucinogens typically disrupt PPI in animals e.g. (Sipes and Geyer, 1994, 1995; Vollenweider et al., 1999; Bubenikova et al., 2005; Palenicek et al., 2007a, 2008, 2010). The potency of PMMA to disrupt PPI is comparable to that of MDMA (Padich et al., 1996; Vollenweider et al., 1999; Bubenikova et al., 2005) and phentehylamine hallucinogens like mescaline or 4-bromo-2,5-dimethoxyphenethylamine (2C-B) (Palenicek et al., 2007a, 2008). Methamphetamine as a related stimulant in studies with mice and rats disrupts this reaction more potently (Mizoguchi et al., 2009; Satow et al., 2009). Interestingly a decrease, though not significant, in ASR was observed after PMMA 5 mg/kg. We observed similar findings with mescaline and 2C-B (Palenicek et al., 2007a, 2008) and others with the serotonin releasing agent fenfluramine (Padich et al., 1996). On the contrary, no change or an increase in startle amplitude has been reported for MDMA (Kehne et al., 1992; Padich et al., 1996; Bubenikova et al., 2005). Thus, the sensorimotor disrupting effect of PMMA is comparable to other psychoactive drugs; however a decreasing trend of ASR might indicate some similarities with drugs like 2C-B or mescaline.

Temperature measurements revealed that only PMMA 5 mg/kg slightly decreased temperature in animals housed separately immediately after administration. In animals housed in groups PMMA 5 mg/kg induced only a minor increase in temperature 3 h after administration. On the contrary, PMMA 20 mg/kg significantly increased temperature in both groups of animals of about 1.5 °C above the control animals. Maximum temperatures were reached at 2.5 h in isolated animals, whereas under crowded conditions the maximum change appeared more rapidly at 2 h and the increase persisted until the end of the measurement. Again comparable data about PMMA are missing throughout the literature. However, several studies have found that PMA increases body/core temperature in rats and that its increase depends on the ambient temperature. In general, the higher the ambient temperature and PMA dose the higher the increase in body/core temperature was observed (Nichols et al., 1975; Jaehne et al., 2005; Freezer et al., 2005; Jaehne et al., 2007; Stanley et al., 2007) with low ambient temperatures also inducing an opposite effect (Daws et al., 2000). Similar findings were described for MDMA (Gordon et al., 1991; Dafters, 1995; Daws et al., 2000; O'Loinsigh et al., 2001; Green et al., 2004; Jaehne et al., 2007), for methamphetamine (Arora et al., 2001; Jaehne et al., 2007; Myles and Sabol, 2008) and for phenethylamine hallucinogen 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Salmi and Ahlenius, 1998; Darvesh and Gudelsky, 2003). On the contrary, tryptamine hallucinogens psilocybin, N,N-diethyllysergamide (LSD), 5-methoxy-diisopropyltryptamine (5-MeO-DIPT) induce hypothermia in rats (Ladefoged, 1973; Williams et al., 2007). It is well established that factors like social interaction lead to higher increases in body temperature after MDMA and methamphetamine (Brown et al., 2003; Brown and Kiyatkin, 2004; Green et al., 2004) but we did not directly find such an effect in PMMA. However, animals housed in groups treated with PMMA 20 mg/kg were perspiring extremely. This indicates induction of extensive thermoregulatory mechanisms that over compensate the increase in body temperature. Taken together with the disability to decrease the temperature to normal during the whole measurement, the social interaction of animals attenuated the ability to eliminate the heat. This is very similar to what happens in the typical club setting where these drugs are used by humans and can be a tracer of increased toxicity. Finally, the similarity with MDMA and stimulants or phenylalkylamine hallucinogens is obvious, but not with tryptamine hallucinogens.

PMMA primarily acts on serotonin and possibly dopamine systems as stated previously. Most of the observed effects correlate with the known effects of mixed serotonin-dopamine releasers like MDMA or amphetamines. Since methamphetamine is more active in inducing dopaminergic-like behavior (hyperlocomotion), PMMA by means of its potency resembles more MDMA. However, both serotonin as well as dopamine might be responsible for the observed effects. The temperature increases are comparable to MDMA as well as stimulant drugs underlying similar mechanisms of action. A direct link between the serotonergic function and thermoregulation has been well established. It is generally accepted that while agonism at serotonin 5-HT_{1A} receptors induces hypothermia (Hjorth, 1985; Hedlund et al., 2004; Ootsuka and Blessing, 2006) agonism at 5-$HT_{2A/C}$ receptors induces an opposite reaction (Salmi and Ahlenius, 1998; Darvesh and Gudelsky, 2003; Ootsuka and Blessing, 2006; Ootsuka et al., 2008). 5-HT_{1A} agonism is also responsible for hypothermic response under normal ambient temperature in MDMA treated animals (Rusyniak et al., 2007, 2008) while 5-HT_{2A} agonism is responsible for the hyperthermic response (Schmidt et al., 1990). The role of 5-$HT_{2A/C}$ receptors in this physiological parameter is also supported by the fact that 5-$HT_{2A/C}$ agonists, phenethylamine hallucinogens like mescaline or DOI, induce similar changes in locomotion, PPI as well as thermoregulation (Sipes and Geyer, 1994, 1995; Salmi and Ahlenius, 1998; Krebs-Thomson et al., 1998a; Darvesh and Gudelsky, 2003; Palenicek et al., 2008).

5. Conclusion

PMMA induced behavioral and physiological changes that are comparable to stimulants, MDMA as well as hallucinogens; these are

overall stimulatory locomotor effects accompanied by initial ataxia with the high dose of the drug and stereotyped circling in the arena, the disruption of sensorimotor gating, and hyperthermia. The dramatic hyperthermic effect in our model is in line with findings for PMA and MDMA. Hyperthermia is a typical symptom of serotonin syndrome in humans. It is associated with PMA poisoning, most likely induced by concomitant inhibition of MAO and increased serotonin release. Our findings indicate PMMA might have a similar toxic potency. Crowded conditions might even increase the risk of PMMA poisoning. The gradual influx of the drug into the brain and delayed achievement of its maximum concentration at higher doses might be responsible for its apparently delayed onset of psychological effects and their severity and duration in humans. Such symptoms are frequently associated with ingestion of high doses / or other drugs by users which in turn increases the risk of serotonin syndrome and toxicity.

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