Pre- and postnatal effects after exposure to the novel psychoactive drug, methylenedioxypyrovalerone (MDPV) in an animal model

Doctoral thesis

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Budapest

2019

1. Introduction

During the last decade, abuse of hundreds of new psychoactive drugs became widespread, largely affecting the illegal drug market and making the job of the authorities and healthcare workers more difficult. Little is known about the short and long-term effects on the developing nervous system of the newly emerged psychoactive drugs, despite the fact that pregnant females may occur among the users.

One of the most widespread group of new psychoactive drugs is that of the synthetic cathinones. These substances are derivatives of the active ingredient of khat *(Catha edulis)*, they belong to the psychostimulants by their primary effects, just like the well-known amphetamine and cocaine. One of the most important member of the synthetic cathinone family is 3,4-methylenedioxypyrovalerone (MDPV). Use of MDPV results in desired effects like increasing performance, anorexia and decreased fatigue, but also in severe side effects as agitation, aggression, psychosis and the general activation of the sympathetic nervous system, including tachycardia, hypertonia, hyperthermia. The consequences of MDPV abuse during pregnancy are practically unknown, however, based on the investigation of similar agent (e.g. cocaine), it is assumable

that is has a negative effect on the outcome of pregnancy and the psychomotoric development of the newborn.

Considering the mechanism of action, MDPV is a competitive inhibitor of dopamine and norepinephrine transporter, in that way it increases their concentration in the synaptic cleft. In laboratory animals MDPV increases locomotor activation, induces stereotypic movements, influences thermoregulation, has a high addiction potential and worsens some cognitive functions.

It is well-known that the psychostimulant amphetamine and methamphetamine have a detrimental effect on maternal adaptation, moreover, cocaine, which has a more similar mechanism of action to the MDPV, injected directly into certain regions of hypothalamus can inhibit maternal behavior, and when given during pregnancy, it inhibits pup retrieval and the building of hide-out nest.

Apart from functional disturbances, structural damage can also be demonstrated after synthetic cathinone exposition. With *in vitro* experiments apoptosis, high release of reactive oxygen species, appearance of autophagic bodies could be demonstrated at the cellular level, depending on the exact substance and its dose. However, MDPV does not cause damage in the striatal dopaminergic endings, nor does it evoke microgliosis. In the very sparse animal experiments,

neurodegeneration could be shown in enthorhinal and perirhinal cortices.

In conclusion, the consequences of the gestational exposure to MPDV are basically unknown, likewise the precise effect of MDPV on the developing brain remains to be clarified. In the present dissertation we are searching for answers to the above stated questions.

2. Aims

In the present dissertation we aimed to answer the following questions:

1) Does the prenatal exposure of MDPV have any effect on the behaviour and development of the offspring?

How does MDPV affect the pregnancy, the parturition and the physical development of the newborn pups?
How does MDPV affect the locomotor activity and the motor coordination of the offspring?

2) Does MDPV have any effect on the maternal adaptation and the maternal care?

Given an impairment of the maternal behavior of MDPV-treated dams, is there any change in the mRNA expression of two relevant hypothalamic neuropeptides, the TIP39 and the amylin?

3) Does MDPV have any neurotoxic effect on adult or developing brain?

If yes, which brain regions are the most affected ones and how severe are the damages?

3. Materials and methods

3.1. Animals and treatment

For the experiments involving prenatal exposure of MDPV we used 40 C57BL/6J female mice, their age ranging between 16 and 22 weeks. The animals were kept in the animal facility of the Semmelweis University, Department of Anatomy under standard laboratory conditions.

The experimental group received 10 mg/kg MDPV in 0.9% sterile saline, by subcutaneous injection from the 8th to the 14th day of pregnancy, the control group received saline.

For the experiments investigating the possible neurotoxic effect of MDPV we injected a single dose of 10 mg/kg MDPV in 0.9% sterile saline intraperitoneally to 7-day-old or adult mice of the same strain (controls received saline only). This young age of 7 days is considered to be equivalent to the third trimester of human pregnancy, which is known as the brain growth spurt.

3.2. Locomotor activity

Locomotor activity was evaluated by placing the mice into the centre of an open field arena (30 × 30 × 30 cm), divided by 3 cm by 3 cm squares, for 10 min. The test was run on the pups of the chronically MDPV-treated mothers at day 7 and day 21, as well as on the mothers themselves.

To further evaluate the movement of 7-day-old pups, we performed force plate actometry, in which the system records the vertical load on a platform where the animal is placed, and the center of gravity (COG) is computed. The signal is digitized onto an X-Y grid, registering a track record of the sway of COG. The platform of the apparatus is divided into 96 quadrangular sectors, therefore the method shows the total travelled distance as well as the occupied area.

3.3. Motor coordination

To assess the motor coordination, we performed grip strength test. The mice were placed on a horizontal non-rotating metal dowel of 3.5 mm diameter, spanning the distance between opposite sides of a square box (side length 25 cm). The animals instinctively hang on to the rod using the front paws, until they lose grip and fall on the bottom. The latency to fall down was measured. The dams and their 21-day-old offspring were tested.

3.4. Maternal behaviour

The nest building behaviour of the pregnant females was tested at three time points: at day 6 of pregnancy (i.e., prior to drug injections), at day 11 (during treatment) and at day 18 (after end of treatment). We placed a pile of pressed cotton at the top of the cage from where the dams could pull the strands

down and build a nest to protect her pups. For measuring the quality of the nest we used a standard 5-point nest-rating scale.

At PD 7 we performed the pup retrieval test with the dams. After removing the dam from the cage, pups were placed in the farthest corner away from the nest. The dam was then returned to the original nest position. The time elapsed between reintroduction of the dam and touching the first pup, as well as the latency to retrieve all the pups to nest position were recorded.

3.6. Evaluation of maternal adaptation with *in situ* hybridization

Regarding the maternal motivation we examined the mRNA expression of two neuromodulator systems: tuberoinfundibular peptide 39 (TIP39) in the posterior intralaminar complex of the thalamus (PIL) and amylin in the medial preoptic area (MPOA). The expression of both neuropeptides increase right after parturition and remains high while the pups are present. The TIP39 containing neurons of the PIL receive afferentation from the somatosensory pathways and partly through amylin expressing neurons in the hypothalamus, contributing to the dams' preference towards pups: the motivational component of the maternal behaviour.

To assess the two neuropeptides, we performed in situ hybridization with autoradiography. Brains of control and MDPV-treated dams were dissected on PD 10, put immediately on dry ice and stored at -80°C until use. Twelve μm thick coronal sections were cut with a cryostat at +0.5 to −0.5 mm (TIP39) or at -2.8 to -3.5 mm (amylin) from the bregma level. Antisense [^{35}S]UTP-labeled riboprobes were used for hybridization at $3*10^5$ dpm/slide. Every 9th coronal section was hybridized to visualize the mRNA expression at 108 μm distances. Following hybridization, RNase A incubation and washes, dried slides were dipped in NTB nuclear emulsion and stored at 4°C for 3 week for autoradiography. Exposed slides were developed and fixed, counterstained with Giemsa and finally coverslipped. During the quantitative analysis mRNA-containing neurons were counted manually on micrographs taken from 3 consecutive coronal sections, in which the TIP39 or amylin signals were found to be most intense. Apart from the cell number, silver grain density over TIP39 or amylin expressing neurons was calculated in each series of brain sections. The resulting figures were normalized for background density.

3.7. Evaluation of apoptotic effect with immunohistochemistry

Twenty-four hours after the treatment of 7-day-old or adult mice we perfused them transcardially with a fixative solution, under terminal anaesthesia, then the brains were dissected. The samples were postfixed in this solution and were stored at 4°C until use, then they were placed in 20% sucrose for 48 hours. Seventy μm thick coronal sections were cut with a freezing microtome. Every second section was collected for caspase 3 immunohistochemistry, while the intermediate sections were processed for Nissl staining. For caspase 3 immunohistochemistry, free-floating sections were washed and pre-treated with pepsin solution for antigen retrieval, followed by a blocking solution of 5% normal goat serum. Then the sections were incubated with a polyclonal rabbit anti-Casp3 antibody (1:1000) for 48 hours, at 4°C. After multiple washes the sections were incubated with Alexa Fluor 488 associated anti-rabbit IgG (1:250) for 3.5 h at room temperature. After short drying, sections were coverslipped and examined under a fluorescent microscope. To evaluate the ratio between the apoptotic cells and the total cell number, selected sections from each series were counterstained with the nuclear marker fluorochrome 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). The number of stained cells in each area of interest was

measured by manual counting with the aid of ImageJ software. Data from 5 sections were collected, representing, together with their alternate Nissl-stained counterparts (which was used to identify certain brain areas), a total of 700 μm tissue thickness in a rostrocaudal direction.

4. Results

4.1. The effect of MDPV on the outcome of parturition

A significant decrease of survival rate was observed among the offspring of MDPV-treated mothers compared to control animals. Whilst all control pregnant females gave live birth at due time, the birth rate of MDPV treated animals was markedly diminished, with further reduction of pup survival due to stillbirth, premature birth, or cannibalism of dams. However, on day 7 there were no difference in the mean body weight of the pups between the two treatment groups.

4.2. Locomotor activation

The offspring of the MDPV treated mothers showed higher locomotor activity in the open field test, both 7 and 21 days after birth. Evaluating the former age group with force plate actometry, we showed, that the increased activity was being present as an increase in the number of areal sectors occupied, rather than an increase of the total distance travelled.

4.3. Motor coordination

On PD 21, we did not find significant difference between the grip strength test results of offspring of MDPV-treated and control mothers.

Seven days after giving-birth, MDPV-treated dams performed in the grip strength test significantly worse than the control animals.

4.4. Evaluation of maternal behaviour

The nest scores of drug treated mothers were significantly lower than those of control animals, when compared during or after treatment.

In the pup retrieval test, although there was no difference between the times elapsed until the first pup was touched, there was a significant increase in the latency to retrieve all pups to the nest, therefore the average time spent returning one pup was longer in the case of MDPV-treated dams.

4.5. Evaluation of maternal adaptation with in situ hybridization

We could not detect any difference between the treatment groups, either in the mean number of TIP39 mRNA expressing cells in the PIL, or in the mRNA densities of the marked cells.

Just like in case of the TIP39, we could not detect any significant difference between the total numbers of amylin

mRNA expressing neurons or between the amylin mRNA densities of the cells in the two treatment groups.

4.6. Apoptotic effect of MDPV

In 7-day-old mouse pups, 24 h after single MDPV injection, we could identify caspase 3 immunoreactive (Casp3+) cells in many telencephalic regions. In several cases, the morphology of the Casp3+ cells resembled a specific group of cells of the given region, which allows a certain level of anatomical identification. Accordingly, in the cortical areas pyramidal or stellate neurons, in the striatum medium spiny neurons showed their typical features. In the MDPV-treated group we detected an increase in the number of apoptotic cells in hippocampus CA1, retrosplenial, cingulate and piriform cortices. The most marked increase of Casp3+ cells was found in the nucleus accumbens. In the above mentioned regions the proportion of Casp3+ cells among the total cell number was significantly higher in the MDPV-treated group, the highest being in the nucleus accumbens (8,89%).

Conversely, we could not detect any difference in the number of apoptotic cells between the treatment groups of adult animals in any brain region.

5. Conclusion and consequences

5.1. Effect of prenatal exposure to MDPV on the outcome of parturition

Prenatal administration of 10 mg/kg b.w. MDPV decreases birth rate, and generally results in the loss of many pups due to a higher frequency of perinatal complications. On PD 7 the mean body weight of the MDPV-treated pups did not differ from the control group, they were developing ostensibly normally.

5.2. Effect of prenatal exposure to MDPV on the behaviour of the pups

The *in utero* drug exposed animals showed higher spontaneous activity compared to controls at the age of 7 days. Both the open field and the force plate actometry tests point in the same direction, i.e., a wider occupancy of space available to the animal, whereas a genuine increase of the distance travelled could not be verified statistically. The travelled distance hardly includes stereotypic movements, which are consequences of dopaminergic activation. Therefore, increased motility of MDPV treated pups means more frequent and erratic deflections in multiple directions, rather than a longer course travelled in a given direction.

We demonstrated in the grip strength test that prenatal exposure to MDPV did not worsen the motor coordination of young animals, however 7 days after giving-birth the MDPV-treated dams showed a decreased latency of losing grip, as compared to control animals. In case of chronic *in utero* drug treatment such effect is barely visible.

5.3. Effect of MDPV exposure during pregnancy on maternal adaptation

The results of the pup retrieval test demonstrated an increase in the latency to retrieve all pups to the nest among the MDPV-treated dams, and the mean time spent to retrieve one pup also showed an increase. There were no difference in the open field results of the dams, therefore it is clear, that slower pup retrieval is not a consequence of altered motility, but of a specific disturbance of maternal motivation. The quality of the nests built during pregnancy was significantly poorer due to the MDPV treatment, which means that the impairment of maternal care does not depend on the presence of pups.

By testing the TIP39 and amylin mRNA expression, we could detect a possible alteration in a functional unit, which is independent from the mesolimbic dopaminergic system, but it still affects maternal motivation. Since neither of these neuropeptides showed an altered level of expression (either in

the number of expressing cells or in the radiographic grain density) in response to MDPV treatment, we can rule out an inadequate processing of somatosensory stimuli from the pups as a possible cause of the disturbance in maternal motivation.

5.4. Apoptotic effect of MDPV

Our results demonstrate that even a single injection of MDPV can induce apoptosis in the 7-day-old mice, however the same does not happen in adult animals. The alterations mainly effect pallial or subpallial brain regions, which play a role in emotions and motivation (nucleus accumbens, hippocampus CA1, retrosplenial and piriform cortices). The ratio of apoptotic cells compared to the total number of cells was found to be the highest in the nucleus accumbens.

6. Publications

6.1. The dissertation is based on the following publications

Ádám Á, **Gerecsei LI**, Lepesi N, Csillag A. (2014) Apoptotic effects of the 'designer drug' methylenedioxypyrovalerone (MDPV) on the neonatal mouse brain. Neurotoxicology, 44**:** 231-236. **IF=3,379**

Gerecsei LI, Csillag A, Zachar G, Gévai L, Simon L, Dobolyi Á, Ádám Á. (2018) Gestational Exposure to the Synthetic Cathinone Methylenedioxypyrovalerone Results in Reduced Maternal Care and Behavioral Alterations in Mouse Pups. Front Neurosci, 12**:** 27. **IF=3,566**

6.2. Other publications

Gerecsei LI, Ádám Á. (2015) A dizájner drog metiléndioxi-pirovaleron hatása a fejlődő idegrendszerre kísérletes állatmodellben. Orv Hetil, 156**:** 1221-1225. **IF=0,291**

Gerecsei LI, Balázsa T, Echevarría D, Ádám Á, Zachar G, Csillag A. (2019) Selective neuronal death following exposure to methylenedioxypyrovalerone (MDPV) is accompanied by an

inhibition of NMDA receptor NR2B subunit expression. Acta Neurobiol Exp, 79:19-27. (közlés alatt) **IF=1,500**