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Selective neuronal death following exposure to methylenedioxypyrovalerone is accompanied by an inhibition of NMDA receptor NR2B subunit expression

László István Gerecsei[1], Tamás Balázsa[1], Diego Echevarría[2], Ágota Ádám[1], Gergely Zachar[1] and András Csillag[1]

[1] *Department of Anatomy, Histology and Embryology, Semmelweis University, Faculty of Medicine, Budapest, Hungary,*
[2] *Institute of Neuroscience (UMH-CSIC), Miguel Hernández University, Alicante, Spain,*
** Email: csillag.andras@med.semmelweis-univ.hu*

In a previous study, methylenedioxypyrovalerone (MDPV), a designer drug of the cathinone family, caused selective enhancement of Caspase3 immunoreactive (Casp3+) apoptotic cells in the nucleus accumbens (NAc) of 7-day-old mice. To further elaborate on the mechanism underlying MDPV-elicited apoptosis, here, we investigated the appearance of Casp3+ cells in developing neural tube explants of E12.5 mice, following MDPV treatment *in vitro*. Apoptotic cells appeared in large number in the pallium as radial progenitor cells and multipolar neurons, and in the subpallium including the future NAc, both in control and MDPV treated specimens. MDPV did not cause gross morphological changes in the neural tube or in the abundance of Casp3+ cells, based on a visual impression, though quantification was not attempted. We also studied the changes in NMDA receptor (NMDAR) protein subunits NR1 and NR2B in the NAc of 7-day-old MDPV treated and control mice, using western blotting of tissue obtained by selective dissection. In MDPV treated animals, expression of NR2B was lower than in the control animals, whereas expression of NR1 did not differ significantly from controls. The findings indicate that, during early postembryonic development, downregulation of the NR2B receptor subunit (at this time predominant in the NMDAR) is accompanied by a decreased viability of neurons. Decreased viability is expressed, in this case, as enhanced susceptibility to stimulation by MDPV – essentially a robust dopaminergic agent, potently affecting the neurons of the NAc. The findings are likely relevant to dopaminergic/NMDAR interactions and a potential pro-survival role of the NR2B subunit in critical phases of neural development.

Key words: apoptosis, basal ganglia, nucleus accumbens, neurodegeneration, dopamine

INTRODUCTION

Synthetic cathinones belong to the group of the so-called novel psychoactive substances (NPS) also known as 'designer drugs'. One member of the family, methylenedioxypyrovalerone (MDPV), is a frequently used psychoactive drug of abuse. In a previous study (Ádám et al., 2014), we found a marked increase in the number of apoptotic cells in the piriform cortex, retrosplenial area, hippocampus CA1 and nucleus accumbens (NAc), without an overall change of cell density, in 7-day-old mice (developmentally comparable to the human fetus of the third trimester). Apoptosis in C57BL/6J mice 24 hours after i.p. injection of 10 mg/kg body weight of MDPV or vehicle (saline) was estimated by anti-caspase 3 (Casp3) immunohistochemistry and cell counting. The greatest percentage of increase in apoptotic cells was observed in the NAc. However, MDPV did not seem to increase apoptosis in adult mice (Ádám et al., 2014). Based on these observations, the

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mechanism of action appears to be developmentally determined.

The objective of the present study was to further elaborate on the factors and potential mechanisms underlying the apoptotic effect of MDPV. First, we addressed the question whether the boost of apoptosis observed as a result of postnatal MDPV administration could also be demonstrated in the embryonic brain. In a previous study, we reported that gestational exposure of pregnant mice to this cathinone in a critical period between E7 and E14 was followed by subtle locomotor dysfunction in the offspring (Gerecsei et al., 2018). This observation raised the possibility of a definite, if by no means destructive, interaction of MDPV with the developing brain. The present study attempts to address this issue further by investigating the effect of direct exposure of the neural tube to MDPV, potentially interfering with programmed cell loss in the course of early development of the neural tube.

Secondly, given the conspicuously high incidence of apoptosis evoked by MDPV in the NAc, specifically implicated in reward and addiction, the neurodegeneration elicited by the drug is likely related to its emotional and rewarding effects. One of the candidate targets are N-methyl-D-aspartate receptors (NMDAR) which are known to undergo developmental alteration (maturation) and also display functional plasticity. We, therefore, investigated the alterations of NMDAR subunit distribution by measuring the expression levels of receptor proteins in the NAc of 7-day-old mice, 24 hours after systemic bolus administration of MDPV.

A plausible mechanistic link between NMDARs and the action of MDPV is the neurotransmitter dopamine. Like other members of the cathinone family, or cocaine and methamphetamine, MDPV is known to exert its effect mainly via dopaminergic activation. A potent blocker of the dopamine transporter and norepinephrine transporter, with a weaker effect on serotonin uptake (Baumann et al., 2013b), MDPV induces an outward current, similar to, but longer lasting than cocaine (Cameron et al., 2013). MDPV is a dose-dependent reinforcer in a continuous self-administration schedule (Watterson et al., 2014), also evoking stereotypies, elevating heart rate, blood pressure and core body temperature (Marusich et al., 2012; Baumann et al., 2013a, Fantegrossi et al., 2013).

Here, we examined whether alterations in NMDAR subunit expression could also play a role in the apoptosis evoked by MDPV. Given the age-dependent shifting of heteromeric NMDARs known as the NR2B to NR2A switch (Gray et al., 2011), and, correspondingly, an expected low level of NR2A in the mouse pups at 7 days of age, we used the expression of NR2B together with NR1, as a general marker of overall NMDAR expression. *Prima facie* it was expected that an enhanced proportion of NR2B in the assembly of the NMDAR tetramer would lead to diminished survival of the affected neuronal progenitors. It is well-established that extrasynaptic (perisynaptic) NMDARs, which become activated mainly by an overflow of glutamate from the synaptic cleft domain, consist of NR1 and NR2B subunits (Momiyama, 2000). Activation of extrasynaptic receptors has been known to cause neuronal death by shutting off the pro-survival CREB pathway and inactivating ERK and its downstream signaling partners (Hardingham et al., 2002). However, at certain developmental stages and receptor locations, NR2B can also play a pro-survival role (Martel et al., 2009). Thus, determining the direction of change in the NMDAR composition following exposure to cathinone drugs may have important consequences and warrants investigation.

METHODS

Animals

BALB/c mice were mated in the animal facility of the Department of Anatomy, Histology and Embryology. Pregnant females were housed individually under standard laboratory conditions (12 hours dark/12 hours light cycle) with food and water available *ad libitum*. Seven pregnant dams were used for the NMDA receptor analysis, and a further 2 for obtaining neural tube explants (see below). From the 7 litters altogether 50 pups were obtained, from which 3 individuals were used for preliminary testing and optimizing protein yield. The pups were randomly selected for control and MDPV treatment from each litter in near-equal numbers. A total of 47 samples from the NAc (left and right hemispheres combined) were used for NR2B analysis, whereas, for NR1 the final number of pooled samples was 12 (6 controls and 6 MDPV treated samples). Once born, the pups were kept together with the mothers until they had reached day 7 of age. Then, a total of 25 animals of either sex from each litter (5-10 pups/litter), were treated with 10 mg/kg MDPV i.p. (diluted in saline), while the rest (22) of the animals received saline only. The dose was selected on the basis of our preliminary behavioral observations in adult mice: it caused visible alteration of behavior (increased locomotor activity, stereotypies) without lethal consequences. It also corresponded to the MDPV concentration applied in previous experiments (Ádám et al., 2014), bringing about enhanced locomotor activity and apoptotic cell death in several brain regions, particularly the NAc, of 7-day-old mice. Following treatment, the pups remained with the dams until they were sacri-

ficed 24 hours after the injection. For embryonic treatments, BALB/c (white) laboratory mice with timed pregnancy were purchased from the National Institute for Oncology, Budapest.

The experiments were conducted in conformity with the laws and regulations controlling experiments and procedures in live animals, as described in the Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985). Our Department is in possession of a valid experimental license for working with laboratory animals including mice, issued by the Food Chain Safety and Animal Health Directorate of the Government Office for Pest County, Hungary (license number: XIV-I-001-2269-4/2012).

Chemicals

The racemic mix of MDPV used was the product of LGC Standards (Teddington, Middlesex, UK) and purchased through Medinspect Kft (Fót, Hungary). The experiments were carried out under a special license (#27924/2011/KÁB) issued by the Narcotic Drugs Control Department, Office of Health Authorization and Administrative Procedures, Hungary.

Western blot analysis of NMDA receptor protein subunits

Immediately after decapitation of the animals, the brains were removed and dissected into coronal slabs with the help of a brain mold. The slabs from the appropriate coordinates were placed on top of a black powder-coated aluminum box containing ice. Tissue samples of the NAc were dissected bilaterally under a stereomicroscope. The diagram in Fig. 1A shows the rostral facet of the cut slab with the approximate borders of dissection. The removed tissue block contained major parts of the NAc (practically the whole core and most of the shell), together with fragments of the adjacent septum, bed nucleus of stria terminalis and the anterior commissure. Given the small brain size of 7-day-old pups, most of the NAc samples weighed less than 10 mg. This presented a technical difficulty in immunoblotting, especially in the case of the anti-NR1 antibody of lesser detection sensitivity. Therefore, we had to use pooled samples for quantification of NR1. For NR2B detection and quantification, the original protein values were sufficient (except for 5 samples which had to be eliminated because the final protein concentration of the lysate fell below the limit of detectability), whereas, NR2A proved to be below the detection level in this age group (see above). Nevertheless, the gain in tissue specificity (samples centered on the NAc) far outweighed the loss in the protein available for Western blot.

The dissected brain specimens (the combined bilateral samples counted as one) were immediately placed into plastic vials and stored in dry ice until further procedures. The specimens were homogenized in ice-cold lysis buffer (150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate, SDS; 50 mM Tris, pH 8.0). Due to the low weight, samples were pooled in the case of NR1 level measurement. To separate the cellular debris from total protein content, centrifugation at 4°C, 1,200 g for 10 minutes followed. The supernatant was centrifuged again at 15,000 g for 10 minutes at 4°C. The sediment contained the crude synaptosome fraction. Protein concentrations in the brain homogenates were determined using the BCA method (Olson and Markwell 2007). Samples were dissolved in Laemmli buffer (Sigma-Aldrich) and heated at 96°C for 5 minutes. Then, all samples were diluted to an even concentration (1 µg/µl). Samples containing 10 or 20 µg of total protein (for NR2B or NR1, respectively) were loaded onto 8% acrylamide gels and processed with BioRad Mini-Protean III vertical electrophoresis and blotting systems. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membranes. Then, the membranes were immediately incubated with 1% skimmed milk (Cell Signaling) in TBS-T buffer (0.05 M Tris-buffered saline pH 7.4 and 0.1% Triton X) for 1 hour at room temperature. The membranes were incubated with anti-NR1 (diluted at 1:1000), anti-NR2B (diluted at 1:2000) or anti-β-actin (1:100,000) primary antibodies (Cell Signaling, rabbit) overnight at 4°C. After washing, HRP conjugated anti-rabbit secondary antibody (DAKO, 1:20,000) was added to the NMDA receptor samples, and HRP conjugated anti-mouse secondary antibody (DAKO, 1:10,000) to the beta-actin samples. The blots were visualized and quantified with an enhanced chemiluminescence detection system (BioRAD) and standardized by the luminosity of the housekeeping protein beta-actin using ImageLab software.

MDPV exposure of E12.5 mouse neural tube explants

Details of the technique have been previously reported (Echevarría et al., 2001). Briefly, the uterine horns of pregnant dams were removed and immediately placed in ice-cold buffer (PBS) solution until further dissection. The embryos were individually dissected from the amniotic sac, the heads were cut off at the level of rhombomere 2 and the neural tube was exposed by careful removal of the epidermis and mes-

enchyme, with the help of a binocular stereomicroscope. Whole mouse telencephalic vesicles including the striatal region, were dissected from E12.5 mouse embryos and incubated in 24-well tissue culture plates (in 400 µl/well) with 4% fetal bovine serum in Neurobasal culture medium with supplement of B-27plus and Penicillin-Streptomycin (100 U/ml – 100 µg/ml; Thermo Fisher Scientific). Cultures were maintained for 24 hours in an incubator at 37°C, with 5% CO_2 and 95% humidity. To study the effect of drug, 0.1 mg/ml of MDPV (n=5), or 1.0 mg/ml of MDPV (n=5) was added to the incubation medium, whereas 6 specimens contained no added drug (control). Following incubation, the specimens were fixed by immersion in 4% buffered paraformaldehyde for 2 hours, washed in PBS, immersed in 10% and 20% sucrose and frozen. From the tissue blocks, 10-20 µm thick sections were cut with a cryostat, transferred onto glass slides and allowed to dry.

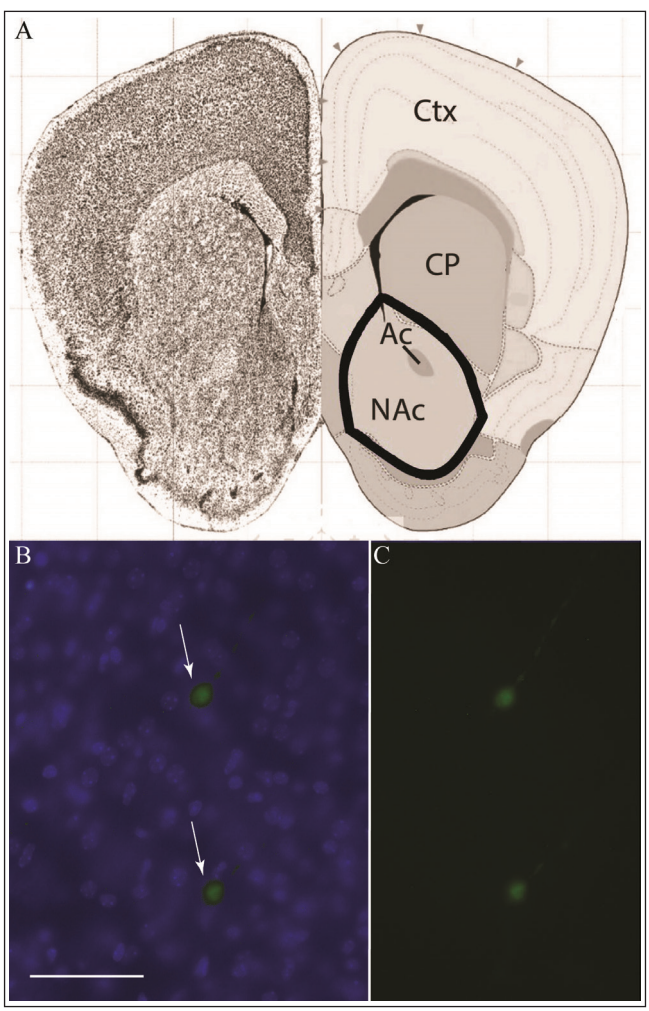

Fig. 1. (A) Diagrammatic representation of the dissection of nucleus accumbens specimens removed for western blotting. The rostral facet of the coronal brain slab, corresponding to the matching coronal Image 41 of the mouse from the Allen Brain Atlas (http: //atlas.brain-map.org), is shown. The position of the NAc, its surrounding regions and their proportions to the whole sectional area correspond well to those observed in a P7 mouse. The frame marks the approximate outlines of the tissue sample. Ac – anterior commissure, CP – caudate-putamen, Ctx – cortex, NAc – nucleus accumbens. (B-C) Representative fluorescent microscopic images depicting two apoptotic (Casp3+) neurons (arrows) against the background of DAPI labelled nuclei in the striatum of a PD7 mouse, 24 hours after treatment with a single i.p. injection of MDPV. (B) Combined image. (C) Single channel image, for the better demonstration of the slightly varicose processes of bipolar-looking (presumably progenitor) neurons. Scale bar: 50 µm.

Immunostaining for activated Caspase-3

For immunohistochemistry, the sections were incubated in a moist chamber under droplets of the following solutions. The sections were washed in 0.1 M PB (4-10 minutes) and pretreated with pepsin solution for antigen retrieval (0.1 mg/ml pepsin with 1 N HCl in dist. H_2O), followed by a blocking solution of 5% normal goat serum (NGS; in 0.1 M PB for 30 minutes at room temperature). Then, the sections were incubated with a polyclonal rabbit anti-Casp3 antibody (Sigma-Aldrich; 1:1000, in PBS containing 1% NGS and 0.3% Triton X-100) for 48 hours at 4-8°C. After rinsing in 0.1 M PB (3 x 20 minutes), the sections were incubated with Alexa Fluor 488 associated anti-rabbit IgG (Molecular Probes; 1:250, in 0.1 M PB) for 3.5 hours at room temperature. Sections were mounted in PBS-glycerol 1:1 and viewed and photographed with an Olympus BX 50 fluorescent microscope equipped with a digital camera. Immunostaining of the postembryonic specimens was carried out on free-floating frozen sections, as reported previously (Ádám et al., 2014). Selected sections were counterstained for 30 minutes at room temperature with the nuclear marker fluorochrome 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich), 5 mg/ml in PBS.

RESULTS

Similar to the previously reported study (Ádám et al., 2014), a considerable number of Casp3+ apoptotic cells were observed after MDPV treatment of 7-day-old neonatal mice, amounting to 1-2% of all cells (as detected by DAPI counterstaining) in most forebrain regions, e.g. the striatum (Fig. 1B). A specific enrichment of apoptotic cells in the NAc has already been reported in detail in the cited study (Ádám et al., 2014), and is not shown here. Casp3+ immunoreactivity clearly marked the outlines of cells of neuronal morphology, although the bulk of the label was accumulated in the nuclear and perikaryal regions (Fig. 1C). The morphological

features of apoptotic cells are well discernible also in embryonic brain specimens (Fig. 2A), clearly marking bipolar progenitor cells as well as multipolar neurons in certain regions of the pallium of the telencephalon (Fig. 2B). Following treatment with MDPV, the overall structure of the neural tube appeared normal. and Casp3+ neural elements were abundant both in pallial and subpallial (including the prospective NAc) regions (Fig. 2C). Control specimens (without MDPV treatment) gave a very similar qualitative impression to those from MDPV treated neural tubes (Fig. 2D-E), both containing a large number of overlapping Casp+ cells. Thus, the embryonic study suggested that MDPV treatment did not affect the gross morphology of the neural tube, nor did it visibly alter the physiological cell loss in the course of early neurogenesis and migration. Next, we turned to the question of whether drug treatment at the age of 7 days, known to elicit a robust increase in apoptotic cells in the NAc (Ádám et al., 2014), would also alter the subunit composition of NMDARs in the same brain region. As detected by western blotting of NMDAR protein components (Fig. 3A-C), the expression of NR2B subunits in the MDPV-treated group was significantly decreased ($F_{3,34}$=8.36, P=0.043). Conversely, the expression of NR1 subunits was not significantly altered (F_{10}=1.47, P=0.253) (Fig. 3C), i.e. an overall downregulation of NMDA receptors in total is not supported by the findings. However, lacking firm data on

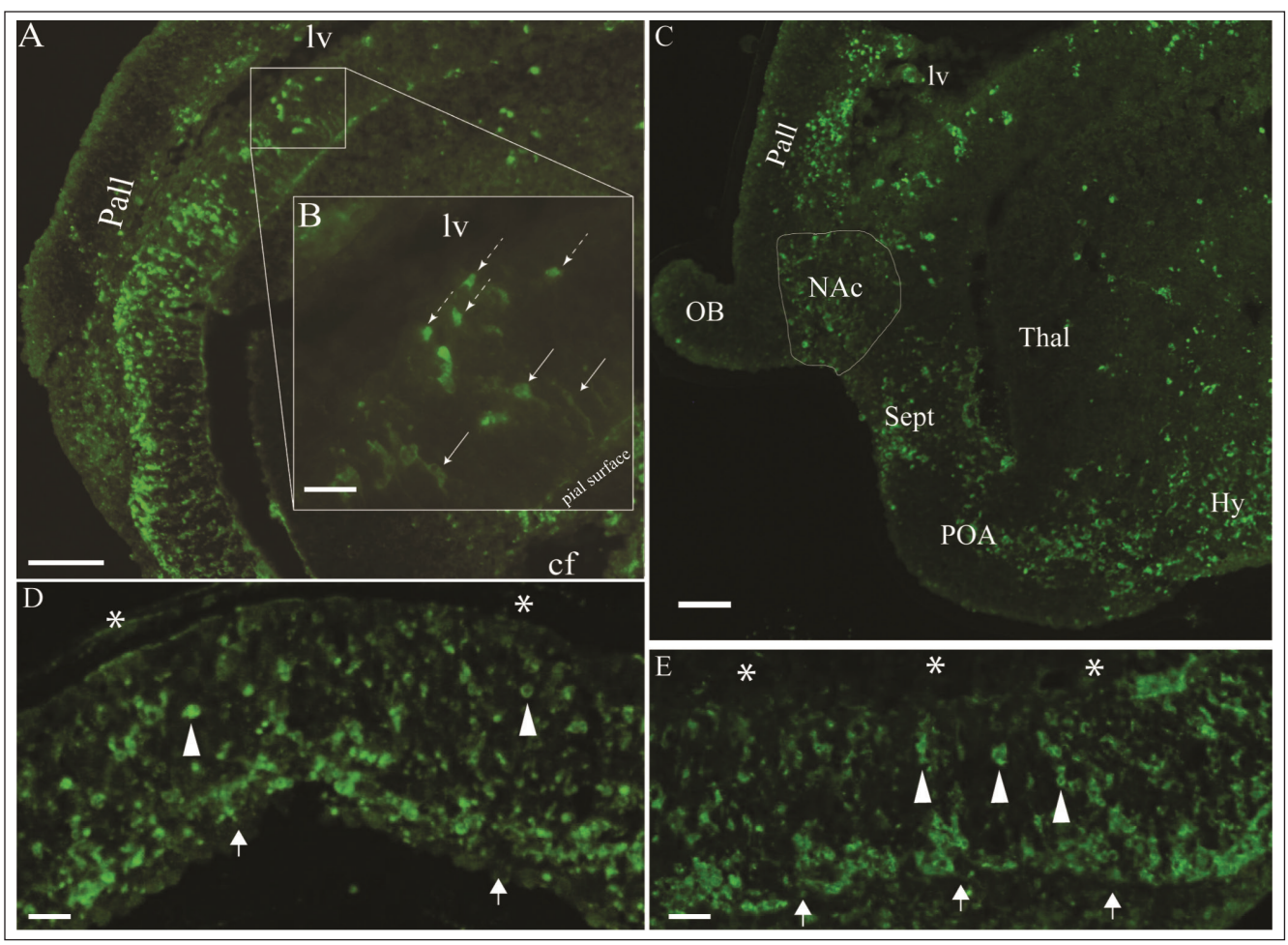

Fig. 2. (A-E) Representative fluorescent microscopic images of the forebrain region of the E12.5 mouse neural tube, directly exposed to 1 mg/ml MDPV in organotypic culture medium for 24 hours, demonstrating the distribution of apoptotic (Casp3+) cells. (A) Overview in sagittal section. (B) An enlarged view of the frame over the pallium shows the types of Casp+ bipolar neural progenitors (arrows) nearer the pial surface and multipolar neurons in the juxtaventricular layer (dashed arrows). (C) Abundance and distribution of apoptotic (Casp3+) cells in pallial. subpallial and diencephalic regions. (D) Representative image of the pallium of a control neural tube. (E) Representative image of the pallium of an MDPV treated neural tube. On (D) and (E) the surface directed toward the telencephalo-diencephalic border is marked by asterisks, whereas, the juxtaventricular surface is marked by bold arrows. Some Casp+ cell bodies are indicated by arrowheads. Note the similar density and overlapping of labeled cells and their processes in these regions of control and MDPV treated pallia. cf – cephalic flexure, lv – lateral ventricle, Hy – hypothalamus, NAc – nucleus accumbens (septostriatal transition area), OB – olfactory bulb, Pall – pallium, POA – preoptic area, Sept – septum, Thal – thalamus. Scale bars: 200 μm (A, C), 50 μm (B, D, E).

the NR2A receptor subunit, a selective downregulation of NR2B would require further confirmation.

DISCUSSION

The cathinone drug MDPV has been reported to cause selective enhancement of Casp3+ (apoptotic) cells in certain limbic forebrain regions, in particular, the NAc of 7-day-old (but not of adult) mice (Ádám et al., 2014). That this neurodegenerative effect, evoked by a bolus injection of the drug to young neonatal mice, might have something to do with a dopaminergic surge (also responsible for other rewarding and addictive effects), has been supported by additional findings on prenatal animals. Chronic MDPV administration during pregnancy resulted in increased locomotor activity of 7- and 21-day-old pups, also causing an impairment of maternal care, however, the latter could not be explained by changes in two relevant hypothalamic peptides (TIP39 and amylin). Thus, the observed anomalies were likely associated with agitated behavior and negligence due to the (mainly dopaminergic) psychoactive drug (Gerecsei et al., 2018).

In the current study, while confirming the presence of apoptotic (Casp3+) cells of distinctly neuronal morphology in the striatum of 7-day-old neonatal mice, we also attempted to investigate if there was a direct neurodegenerative effect of MDPV in the course of the early development of the neural tube. Apoptotic cells appeared in large number already at postnatal day (PD) 12-13 in mouse embryonic brain explants, both in pallial zones (radial cells and multipolar neurons) and in the developing subpallium including the future NAc. Such an abundance of Casp3+ cells did not make it possible to evaluate a direct effect of MDPV on embryonic brain explants since any subtle changes brought about by MDPV treatment would have been masked by the massive surge of apoptosis in control specimens. However, it did suggest that MDPV did not affect the gross morphology of the neural tube or alter the physiological cell loss in the course of early neurogenesis and migration. This period corresponds to the first wave of apoptosis (Blaschke et al., 1998), where the surge of apoptotic cells in the embryonic brain precedes the phase of synaptogenesis in the mouse (notably, in humans, the period of synaptogenesis sets in well before birth, in the third trimester of gestation). In prenatal

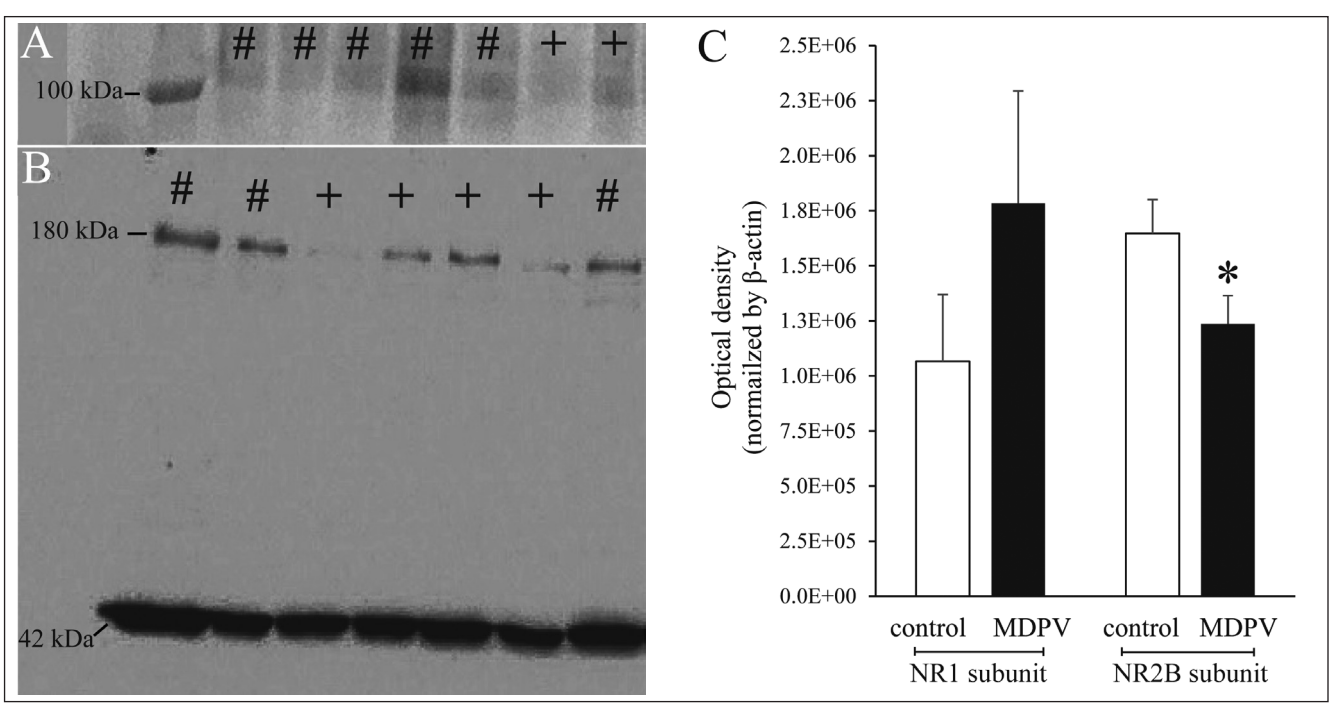

Fig. 3. Abundance and distribution of NMDAR subunit proteins determined by quantitative western blotting from selective mouse brain samples enriched in the nucleus accumbens, as a function of treatment with MDPV. (A) Representative immunoblot images of NR1 at 110 kDa. The pockets marked by (#) represent control (saline-treated animals), whereas, those marked by (+) represent MDPV treated animals. (B) Representative immunoblot images of NR2B at 180 kDa visible above those of the housekeeping protein beta-actin at 42 kDa. The pockets marked by (#) represent control (saline-treated animals), whereas those marked by (+) represent MDPV treated animals. (C) Diagrams of optical density values (normalized by actin) for NR2B and NR1 receptor subunit proteins. The columns show the mean + S.E.M. The values of n for NR1: n=6, 6 (for MDPV and control, respectively), and those for NR2B: n=21, 21 (for MDPV and control, respectively). The asterisk represents a statistically significant difference $p<0.05$.

mice, the amount of Casp3+ cells were found to peak at E14.5 (Renier et al., 2014), i.e. quite close to the period of our current study. Such robust apoptosis in the embryonic brain, amounting to 50 percent of total cells in rodents (Blanquie et al., 2017) may sound surprising and certainly is not yet fully understood. However, programmed cell death most likely subserves the selection of the appropriate clones of neurons. Furthermore, activity patterns strongly determine the fate (survival or apoptosis) of maturing neurons, as reported for cortical areas of early neonatal mice (Blanquie et al., 2017).

Expression of activated Casp3, representing the second surge of apoptosis (Southwell et al., 2012; Nikolić et al., 2013), can also be observed in the intact postembryonic brain as an indicator of physiological cell loss. In the mouse primary motor and somatosensory cortices, the density of apoptotic cells (as revealed by Casp3 immunostaining) was found to peak between PD 4 and PD 7, the upper cortical layers (I-IV) displaying the highest values (Blanquie et al., 2017). Apoptotic (Casp3+) cells were found to be particularly abundant in the NAc of 4-day-old mice, as compared to other regions of the basal forebrain (bed nucleus of the stria terminalis, caudate-putamen), only exceeded by the medial preoptic area (Broad et al., 2009). Mice have been found to be susceptible also to the apoptotic neurodegeneration induced by ethanol, at the end of the first postnatal week (the synaptogenesis period) (Olney et al., 2002).

Based both on the proposed molecular (dopaminergic) mechanism and on the observed age dependence of the drug effect, it was reasonable to assume that the neurodegenerative action of MDPV is likely related, at least in part, to alterations in NMDAR subunit composition.

The subunit composition of heteromeric NMDARs undergoes dynamic changes during pre- and postnatal development of rodents. The most important postnatal change is known as the NR2B to NR2A switch, as a result of which part of the former subunit will get gradually replaced by the latter (Gray et al., 2011). In a detailed study from postsynaptic density (PSD) enriched hippocampal samples of postnatal mice, the NR2B/NR2A ratio was found to be over 1.5 at day 7, just under 1.0 at day 14 and ca. 0.8 at day 28, with a further slow decrease till day 56 (Sinclair et al., 2016). This change is due mainly to a robust increase in the abundance of GluN2A, whereas, GluN2B remains largely stable (Sinclair et al., 2016). The NMDARs containing the subunit 2B participate mainly in neurogenetic signaling, in particular, the development and potentiation of synapses (El Gaamouch et al., 2012), even in the wiring of adult-born neurons of the olfactory bulb (Kelsch et al., 2012). Those NMDARs harboring the subunit 2A will be operational in synaptic transmission, e.g. the generation of excitatory postsynaptic current (EPSC). However, there is evidence to suggest that, even in the adult brain, both NR2A and NR2B can be present and functionally active concurrently, in particular in the NAc (Chergui, 2011). Although recent studies have revealed an increasing contribution of triheteromeric NMDARs, containing two NR1 subunits alongside one NR2A and one NR2B (Zamzow et al., 2013; Stroebel et al., 2018), or NMDARs with other NR2 subunit forms (C and D), the reported percentage of the 2A or 2B subunits among functional NMDARs in the NAc was at least 30-34% or 46-57%, respectively (Chergui, 2011). Moreover, NR2A and NR2B proved to be the only GluN2 subunits to account for the NMDAR-EPSCs in the CA1 of the hippocampus (Gray et al., 2011).

The findings indicate that under conditions of early postembryonic development, downregulation of the NR2B receptor subunit (at this time the main component of the functional NMDA receptor tetramer) is accompanied by decreased viability of neurons (similar to the observation on rat forebrain culture – Fu et al., 2011). In adults (by which time the NR2A subunit becomes predominant in the functional NMDA receptor) no such consequence was seen (Ádám et al., 2014). Decreased viability was expressed, in our case, as enhanced susceptibility to stimulation by MDPV – essentially a robust dopaminergic agent, predominantly affecting the neurons of the NAc. Potential interaction between dopamine and NMDA receptors may be due (as described in an elegant study by Liu et al., 2006) to a direct association between the dopamine D2 receptor and the NR2B receptors (specifically responsive to cocaine), in the postsynaptic microdomain of a certain category (striatopallidal) of striatal/NAc medium spiny neurons. On powerful dopaminergic stimulation (which occurs with a number of psychostimulatory addictive drugs, including MDPV) this D2R-NR2B interaction may lead to an inhibition of NMDAR (via D2R dependent reduction of CaMKII activity and NMDA phosphorylation) and the suppression of the indirect striatal pathway, leading to hypermotility (see a recent study by Giannotti et al., 2017). Selective vulnerability of striatal neurons to neurotoxic damage due to methamphetamine exposure (ultimately evoked by excess dopamine) has been reported previously (Zhu et al., 2006; Tulloch et al., 2011).

Activation of the extrasynaptic (perisynaptic) NMDARs, predominantly consisting of NR1 and NR2B subunits, is known to cause neuronal death by shutting down of the pro-survival CREB pathway and inactivating ERK and its downstream signaling partners (Hardingham et al., 2002). However, in certain developmental

stages and receptor location, NR2B can play a pro-survival role as well (Martel et al., 2009). As evidenced by the present observation, this may well be the case with neonatal mice at the peak of the second apoptotic surge and before the completion of synaptogenesis.

Apart from dopaminergic/NMDAR interactions, other molecular mechanisms evoked by MDPV and potentially causing cell death should also be taken into consideration. A single exposure to MDPV and 1-phenyl-2-(pyrrolidin-1-yl)pentan-1-one (α-PVP) has been reported to change the baseline neuronal activity (expression of the immediate early genes c-Fos and Arc/Arg 3.1 mRNA), both in the striatum and in the hippocampus of mice (Giannotti et al., 2017). Survival of embryonic brain cells may depend on baseline activity (Blanquie et al., 2017). Moreover, a potential link between cathinone (methylone and MDPV) induced apoptosis and autophagy in human dopaminergic cells has also been reported (Valente et al., 2017).

CONCLUSIONS

Apoptotic (Casp3+) cells had appeared in large number already at E12.5 in mouse brain explants, both in pallial zones (as radial cells and multipolar neurons) and in the subpallium including the future NAc. *In vitro* treatment with the cathinone drug MDPV (0.1 or 1 mg/ml) failed to alter the morphology of the neural tube. Massive overproduction of Casp3+ cells in the control brain did not allow evaluation of a direct effect of MDPV on embryonic brain explants.

As a potential molecular mechanism, we studied the alterations of NMDA receptor protein subunits NR1 and NR2B in 7-day-old MDPV treated and control mice, using western blotting from selectively dissected brain samples enriched in the NAc. In MDPV treated animals, the level of NR2B was significantly lower than in control animals, whereas NR1 was not significantly different from controls (i.e. an overall decrease of NMDA receptor expression is unlikely).

The findings indicate that under conditions of early postnatal development, downregulation of the NR2B receptor subunit (at this time the main component of the functional NMDA receptor tetramer) is accompanied by decreased viability of neurons, which is expressed, in our case, as enhanced susceptibility to stimulation by MDPV – essentially a robust dopaminergic agent, predominantly affecting the neurons of the NAc as a main reward center.

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