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Neonatal propofol anesthesia modifies activity-dependent processes and induces transient hyperlocomotor response to d-amphetamine during adolescence in rats

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ABSTRACT

This study examined the influence of propofol anesthesia on the expression of activity-regulated molecules (BDNF and c-Fos) and synaptic plasticity markers (synaptophysin, GAP-43, drebrin) in the frontal cortex and thalamus of 7-day-old (P7) rats. Although these brain regions are the main targets of anesthetic action, they are contained in the cortico-striato-thalamo-cortical feedback loops, involved in naturally occurring and drug-induced psychoses. Therefore,

functional integrity of these loops was examined in adolescent and adult rats through d-amphetamine-induced hyperactivity. Propofol treatment (25 mg/kg) decreased exon-specific and total BDNF mRNA expression in the frontal cortex and thalamus, in a time-dependent manner. BDNF protein level was increased in the frontal cortex and decreased in the thalamus, which was accompanied by the change of phospho-TrkB expression. Similarly to BDNF, the expression of c-Fos was decreased in the frontal cortex while it was changed only at the protein level in the thalamus. Synaptic plasticity markers changed in a time- and region-specific manner, indicating increased synaptogenesis in the frontal cortex and synapse elimination in the thalamus in P7 rats after the propofol anesthesia exposure. These early molecular changes were followed by time-related, increased motor reaction to d-amphetamine in adolescent, but not in adult rats. Our study revealed that exposure of immature brain to propofol anesthesia during the critical phase of development provoked immediate changes in activity-dependent processes and synaptic adjustment, influencing brain capacity to integrate later developmental events and resulting in temporary altered response to acute psychotropic stimulation during adolescence.

KEY WORDS: propofol anesthesia; BDNF transcripts; Trk-B; c-Fos; synaptophysin; locomotor activity

HIGHLIGHTS

- Propofol affected activity-regulated events in the frontal cortex and thalamus of P7 rats
- Propofol changed BDNF and c-Fos expression in time- and region-specific manner
- The treatment influenced the expression of synaptophysin, GAP-43 and drebrin
- The treatment influenced locomotor response of P35 animals to d-amphetamine
- Neonatal anesthesia affected integrity/maturation of cortico-subcortical paths

1. INTRODUCTION

The exposure of infants to general anesthesia is a common practice in pediatric medicine. The evidence from animal studies indicates that early exposure to anesthetics, i.e., before completion of synaptogenesis, can result in widespread apoptotic neuronal degeneration and decreased synaptogenesis, resulting in permanently impaired cognitive development (Jevtovic-Todorovic et al., 2003). Anesthesia-induced neurotoxicity is highly age-related, with the peak around postnatal day (P) 7 (Lu et al., 2006; Pesic et al., 2009; Popic et al., 2012). Although synaptic remodeling has been added to the list of potential mechanisms through which anesthetics could induce long-lasting changes in the developing brain, the baseline of this phenomenon is still unknown (De Roo et al., 2009). The impairment of neuronal activity is crucial for clinically relevant action of general anesthetics, but it also contributes to changes in activity-dependent synaptic plasticity refinement, which is of important for proper circuitry assembly during synaptogenesis.

Our previous study showed that even short-term exposure of neonatal rats to propofol, a frequently used non-barbiturate anesthetic agent, triggered apoptosis in the cortex and thalamus, brain regions that are involved in clinically relevant actions of propofol (Pesic et al., 2009). However, changes in the expression of activity-dependent genes and messengers from synapse-to-nucleus signaling pathways that contribute to the conversion of short-termed signals into

longer lasting changes have not been examined in this model. Important question that remained unanswered is whether in neonatal brain anesthesia-induced changes in neuronal activity, which have been suggested to be a signal *per se* for alterations in both neuronal viability and morphology (De Roo et al., 2009; Lu et al., 2006), are a unique signal for all activity-regulated events to be silenced or there is a certain selectivity. For instance, propofol-induced changes in BDNF gene expression, which is one of the first non-transcription factor effector genes found to be regulated by neuronal activity, have not been examined in detail although several reports pointed to the changes in BDNF protein expression (Karen et al., 2013; Lu et al., 2006; Ponten et al., 2011). The gene for BDNF is comprised of distinct promoters that initiate the transcription of multiple mRNA transcripts, each of which contains an alternative 5′ exon which is spliced to a common 3′ coding exon that contains the entire open reading frame for the BDNF protein (Aid et al., 2007). The influence of anesthesia exposure on c-Fos gene expression is also largely unexplored, although it is widely used for screening neuronal responses to various stimuli and may play an active role in regulating both neuronal survival and synaptic plasticity (Shaulian and Karin, 2002). Moreover, c-Fos activates phospholipid synthesis and is required for neurite elongation through a mechanism independent of its genomic activity (Caputto et al., 2014). Several

in vitro studies examined the role of c-Fos in transcription-dependent mechanisms of propofol action, but the results are opposing (Fibuch and Wang, 2007; Kidambi et al., 2010).

The response of the brain to anesthesia includes both immediate cellular and circuits' responses to the treatment and active emergence from general anesthesia. Namely, it has been shown that propofol changes neuronal activity by reducing glutamate level and enhancing GABA level in motor and sensory cortical areas, thalamus, hippocampus and basal ganglia, with structure-specific degrees in change of glutamate/GABA (Zhang et al., 2009). Also, dopamine released by ventral tegmental area neurons, which send projections to both cortical and subcortical brain regions, could be involved in the emergence from propofol general anesthesia (Solt et al. 2014). This information is of great significance, as the safe and fast emergence from anesthesia, mediated by dopaminergic transmission came recently into the focus of research in the field. Altogether, these data indicate that propofol exposure during neonatal period has a complex neurochemical influence and could be viewed as early challenge to glutamate, GABA and dopaminergic systems in the cortex and thalamus. Although these cerebral structures are essential for consciousness and are therefore

the main targets of anesthetic action, they are contained in the cortico-striato-thalamo-cortical feedback loops that control different behaviors, including naturally occurring and drug-induced psychoses (Paus et al., 2008).

Since in the clinical practice propofol is used mainly for the induction of anesthesia as a single bolus, and is followed by application of other, mainly volatile anesthetics for anesthesia maintenance, there is a need for deliberate assessment of consequences induced by early short-term propofol treatment. In this study we set out to address the influence of single propofol dose on BDNF and c-Fos gene and protein expression. We have also examined TrkB as mBDNF receptor and synaptophysin, GAP-43 and drebrin as well known markers of synaptic plasticity. In order to establish whether transient propofol treatment have a long-term consequences on the integrity of cortico-subcortical circuits, and to what extent these changes remain, we assessed psychomotor response of adolescent and adult animals (propofol pretreated at P7) to d-amphetamine as a challenge, considering the importance of thalamocortical signaling for amphetamine-induced motor activity (Mabrouk et al., 2014).

2. MATERIALS AND METHODS

2.1. Animals

Seven-day-old (P7) Wistar male rat pups with an average body weight of 14 g were used in the experiments. All efforts were made to minimize the suffering of the animals and the number of rats used. In behavioral experiments P35 and P90 male rats exposed to propofol anesthesia at P7 were tested. All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and was approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

2.2. Drugs

Propofol (Recofol®), manufactured for i.v. human use, was obtained from Schering (Turku, Finland). The dose of 25 mg/kg of propofol was chosen based on our previous experiments (Pesic et al., 2009). The ampoules were shaken well and the drug was used according to the manufacturer’s instructions.

D-Amphetamine sulfate (Sigma-ALDRICH Chemie, Germany) was dissolved in saline at a concentration of 1.5 mg/ml and administered i.p. in the dose of 1.5 mg/kg. The drug was chosen to test the integrity of cortico-striato-thalamo-cortical loops, considering novel data about the importance of thalamocortical signaling for amphetamine-induced motor activity (Mabrouk et al., 2014).

2.3. Experimental design

Male rat pups (P7) were separated from their mothers and placed in a temperature-controlled incubator set to an ambient temperature of 35–36°C. Animals not intended to be killed immediately after propofol-induced anesthesia (25 mg/kg) were allowed to recover in the incubator and were returned to their mothers to feed. Loss of the righting reflex (LRR) served as an indicator of anesthetic-induced unconsciousness and sleeping time. Anesthesia-related mortality of neonatal animals treated with the propofol in the dose of 25 mg/kg was not detected; the righting reflex was impaired for 55±5 min (Pesic et al., 2009).

Several techniques were used in the study: Western blotting (n=28 animals), PCR analysis (n=28 animals), immunohistochemistry (n=6 animals) and behavioral testing (n=48 animals).

Western blot and PCR analyses were done on neonatal (P7) animals within hours (0 (control), 1, 2, 4, 8, 16 and 24 h, n=4 per time point) after propofol treatment. We have used the frontal cortex and thalamus for investigation. To avoid eventual effects of laterality the right and left part of examined structures were collected from the same animal, homogenized and further used for molecular analyses. The procedure was consistently performed during the sampling. The samples were stored at -80°C.

For immunohistochemical study the whole brains were isolated from control (n=3) and propofol-exposed pups 4 h after the treatment (n=3 per time point).

Behavioral testing was performed on adolescent and adult animals. They were neonatally exposed to either propofol treatment (25 mg/kg; n=24 per group) or to saline (0.9% NaCl, 2.5 ml/kg; n=24), allowed to recover, returned to their mothers and weaned on P21. On P35 n=12 propofol-injected animals and n=12 saline-injected animals were tested: six animals from each group received saline (1 ml/kg, i.p.), while the other six received d-amphetamine (1.5

mg/ml/kg). The rest of the animals were tested on P90, by the same schedule as described for P35 animals. Experiments were performed between 9:00 and 14:00. Motor activity measurements were initiated immediately after d-amphetamine or saline injection and lasted 120 min. Each animal was tested only once. Before registration, the rats were habituated to the experimental cages for 40 min.

2.4. RNA isolation, reverse transcription and semi-quantitative PCR

Total RNA was isolated from tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Tissue samples were homogenized at a ratio of 1 ml reagent: 0.1 g tissue. RNA integrity was assessed by electrophoresis in a 1% agarose gel. Total RNA was treated with 10 U of RN*ase* A free DN*ase* I and dissolved in diethylpyrocarbonate (DEPC)-treated water.

Reverse transcription (RT) reactions were performed with 5 μg of total RNA, using oligo-dT primers and M-MLV Reverse Transcriptase according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). PCR reactions were performed in pairs since a reference gene for the normalization of target gene expression was included. 200 ng of cDNA were used for each reaction. PCR reactions were performed in the GeneAmp® PCR System 9700

(Applied Biosystems). All PCR reactions were performed from two independent RT reactions. The PCR products were separated in 2% agarose gels stained with ethidium bromide and photographed under UV light. Multi-Analyst/PC Software Image Analysis System (Bio-Rad Gel Doc 1000) was used for densitometry analysis.

Total BDNF mRNA and specific exon-containing BDNF mRNAs were measured by semi-quantitative PCR. We first performed an experiment in order to obtain the patterns of brain structure-specific expression of BDNF mRNA-containing exons I, II, III, IV, V, VI, VII, VIII and IXA (Aid et al., 2007). The reference gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The experimental conditions for PCR were as follows: one cycle at 94°C for 5 min, 35 cycles at 94°C for 15 s, at 56°C for 30 s and at 72°C for 30 s, and at 4°C indefinitely. Obtained results showed that exon IV- and exon VI-containing transcripts were highly expressed in both the frontal cortex and thalamus of P7 rats. Moreover, fainter bands corresponding to exon I-, II- and III-containing transcripts were detected in the thalamus, pointing that at P7 thalamus exhibited more diversity of exon-specific BDNF mRNAs than the frontal cortex (data not shown). The primer sequences of the BDNF transcripts IV and VI that were further investigated and PCR conditions of are provided in Table 1. Changes in total BDNF mRNA expression were detected

with primers to coding exon IX which is common to all transcripts. For these PCRs the reference gene was β-actin, as well as for c-Fos.

2.5. Reverse transcription and quantitative RT-PCR

Reverse transcription reactions were performed in 20 µl using a High-Capacity cDNA Archive Kit according, to the manufacturer's instructions. The reactions were carried out under RNase-free conditions at 25^{o}C for 10 min, and at 37^{o}C for 2 h. The cDNA was stored at -20^{o}C until further use. Relative quantification of synaptophysin mRNA was performed by Real Time RT-PCR using the TaqMan assay (ID Rn00561986_m1, Applied Biosystems). GAPDH was included as an endogenous control to correct for differences in inters assay amplification efficiency (ID Rn00565598_m1), since validation of endogenous control genes showed the stable expression of both GAPDH and β-actin. Each sample was run in triplicate and the mean values of each Ct were used for further calculations. Quantification was performed by the $2^{-\Delta\Delta Ct}$ method. The results obtained by RT-PCR were analyzed by RQ Study Add

ON software for the 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System), with a confidence level of 95% ($p < 0.05$).

2.6. Protein isolation and Western blot analysis

To prepare total protein extract the tissues were homogenized and sonicated in 10 volumes of RIPA buffer containing protease and phosphatase inhibitors. After 30 min incubation on ice, the lysates were centrifuged at 14,000×*g* for 30 min at 4°C. The supernatants were collected and stored at -80°C until required.

Protein concentrations were determined by the bicinchoninic acid micro-protein assay (Micro BCA Protein Assay Kit; Pierce Inc., Rockford, USA), with albumin as standard. Thirty micrograms of the protein extracts were heat-denaturated for 5 min at 95°C in Leammli's sample loading buffer, separated on 10% SDS polyacrylamide gels by electrophoresis, and electro-transferred onto PVDF membranes. Nonspecific protein binding was prevented by treating the membranes with blocking buffer containing 5% nonfat dry milk in Tris-buffered saline/0.1% Tween-20 for 1 h at room temperature. The membranes were incubated overnight at 4°C with primary antibodies diluted in a blocking

buffer. The following primary antibodies were used: anti-synaptophysin (a gift from Dr Reinhard Jahn from the Max Planck Institute, Gottingen, DE), anti-BDNF (Santa Cruz Biotech., sc-546), ant-Trk B (Santa Cruz Biotech., sc-12), anti-TrkB (phospho Y816) (Abcam, ab75173), anti-cFos (Santa Cruz Biotech., sc-52), anti-β-actin (Sigma, A-5316). Samples were incubated with secondary antibodies HRP-conjugated bovine anti-rabbit (Santa Cruz Biotechnology, sc-546), and rabbit anti-mouse (Dako, P0260) in Tris-buffered saline/0.1% Tween-20 for 60 min at room temperature. Three washes with 0.3% Tween-20 in Tris-buffered saline were performed between all steps. The signal was detected by enhanced chemiluminescence and subsequent exposure on an X-ray film. All films were densitometrically analyzed using the computerized image analysis program, ImageQuant 5.0. The optical density of each band was normalized to the corresponding β-actin band.

2.7. Tissue preparation and immunohistochemical analysis

Whole brains of P7 pups were collected at 0 h (control) and 4 h after propofol treatment. Following decapitation, brains were quickly removed and fixed in 4% paraformaldehyde for 12 h and were transferred into graded

(10, 20 and 30%) sucrose for cryoprotection. Brains were frozen in 2-methyl butane and kept at −80 °C until sectioning on a cryotome. The brains were cut in coronal sections 30 μm thick and mounted on Superfrost® glass slides, dried for 12 h at room temperature and stored at −20 °C until staining.

For identification of neuronal cells we used a mouse monoclonal antibody raised against the specific marker of neurons, NeuN (1:500, Chemicon). To examine co-expression with c-Fos rabbit polyclonal antibody (1:100, Santa Cruz, sc-52) was used. All antibodies (primary and secondary) were diluted in PBS. Tissue slices were allowed to warm to room temperature for 2 h and were washed in PBS two times for 5 min. To reduce fixative-induced autofluorescence, incubation in 1% glycine in PBS for 10 min at room temperature was performed. From this step onwards, the samples were washed three times for 5 min in PBS. Antigen retrieval was performed in sodium citrate buffer (10 mM sodium citrate, pH 6.0). Reduction of nonspecific staining was achieved by incubation with 5% NGS diluted in PBS. The slices were incubated with primary antibodies overnight at 4°C, washed and subsequently incubated with goat anti-rabbit (Alexa 488, Invitrogen, 1:500) for c-Fos and goat anti-mouse (Alexa 568, Invitrogen, 1:250) for NeuN, for 2 h at room temperature. The slices were washed in PBS for 1 h, with changes of PBS every 5

min, and mounted with mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., USA). For the negative control, sections were incubated with appropriate secondary antibody without the primary antibody.

Staining was visualized with a Carl Zeiss AxioVision microscope. All images were acquired with 40× magnification. All the sections were stained and analyzed under the same conditions and the same time through visual, qualitative observation by the person who was familiar with the technique and not familiar with the experimental design.

2.8. Measurement of motor activity

The motor activity of rats was recorded individually for each animal in Opto-Varimex cages (Columbus Instruments, OH) that were linked on-line to an IBM-PC compatible computer. The open fields were placed in a light- and sound-attenuated room provided with indirect and homogenous illumination. Each cage (44.2×43.2×20 cm) was equipped with 15 infrared emitters that were located on the x and y axes. An equivalent number of receivers were

located on the opposite walls of the cage. Data were analyzed using Auto-Track software (Columbus Instruments). The Auto-Track interface collects data from the Opto-Varimex unit every 1/10th of a second and categorizes the activity. Locomotor activity was defined as a trespass of three consecutive photo-beams.

2.9. Statistical analysis

The data were statistically analyzed using Statistica 6.0 software (StatSoft Inc.). All descriptive statistics were performed using relative values. The changes in mRNA and protein levels are presented graphically, as percentages (mean±SEM, n=4 per group) of the control samples assumed to be 100%. The data were analyzed by Kruskal-Wallis ANOVA, and if the test resulted in p-value less than 0.05, subsequent comparisons with the control group were performed with the Mann-Whitney *U* test. Significance was accepted at $p<0.05$.

The data for locomotor activity are presented as mean±SEM (n=6 per group) for total activity during 30 min periods after saline or AMPH injection. Before analysis, the data were transformed (sqrt) and analyzed by two-way ANOVA with the propofol anesthesia exposure and time after the saline or AMPH treatment (0-30, 31-60, 61-90, 91-

120 min; repeated measure) as factors. The Fisher LSD test was used for subsequent comparisons between the groups. Significance was accepted at $p<0.05$.

3. RESULTS

3.1. Propofol anesthesia attenuates exon IV, exon VI and total BDNF mRNA expression in the frontal cortex and thalamus of P7 rats

To examine whether short-term propofol anesthesia influenced exon-specific BDNF mRNA expression, we performed RT-PCR analysis of exon IV- and exon VI-containing mRNAs in the frontal cortex and thalamus of P7 rats, which were shown to be highly responsive to changes in neuronal activity (Fig. 1).

Compared to the control group, gradual decreases in exon IV- and exon VI-containing mRNA levels were observed in the frontal cortex (Figs. 1A and 1C; *p<0.05) at almost all time points, while the largest decrease was detected from 2 to 8 h after the treatment (50-70%). In the thalamus of P7 rats significant decreases (30-40%) of both exon IV- and exon VI-containing transcripts were detected at 2 and 4 h post-treatment (Figs. 1B and 1D; *p<0.05

compared to the 0 h time point). The treatment induced decrease in the expression of exon I, exon II and exon III transcripts in the thalamus, as well (data not shown).

To summarize the influence of propofol anesthesia on BDNF gene expression in the frontal cortex and thalamus of P7 rats, we used primers for the coding exon IX which is common to all BDNF transcripts. Compared to the control group, a low level of total BDNF mRNA expression (20-70%) was detected in the frontal cortex of P7 rats at all of the time points examined (Fig. 1E; *$p<0.05$) while in the thalamus a decrease (30-50%) was observed during the 2-8 h after the treatment (Fig. 1F; *$p<0.05$). Changes in total BDNF mRNA expression are largely in agreement with the results obtained for exon IV- and exon VI-containing transcripts.

3.2. The effect of propofol anesthesia on the level of BDNF, total TrkB and phosphorylated TrkB protein expression

Propofol anesthesia induced significant but opposing changes in mature BDNF protein (14 kDa) expression in the frontal cortex and thalamus of P7 rats (Figs. 2A and 2B). In comparison to the control group, significant increase (30-40%) in BDNF level was detected in the frontal cortex 1-4 h after the treatment (Fig. 2A; *$p<0.05$, U-test), while significant decrease (20-40%) in BDNF level was observed in the thalamus 1-8 h after the treatment (Fig. 2B; *$p<0.05$, U-test).

Since BDNF expression was affected by the propofol treatment, we next examined whether these changes influenced the expression of its receptor, TrkB (140 kDa). No significant changes were detected in the expression of total TrkB receptor, either in the frontal cortex or in the thalamus (Figs. 2C and 2D). However, Western blot analysis revealed a significant increase (30%) in phospho-TrkB expression during the 1–16 h post treatment period in the frontal cortex (Fig. 2C; *$p<0.05$) and significant decrease at 4 h post treatment in the thalamus (Fig. 2D; *$p<0.05$), in comparison to the control.

3.3. The effect of propofol anesthesia on the level of c-Fos mRNA and protein expression

To get better insight into the influence of short-term propofol anesthesia on activity regulated processes we have examined changes in c-Fos expression (Fig. 3). Biphasic decrease in c-Fos mRNA expression was detected in the frontal cortex, i.e. during 1-2 h and at 8 h of post anesthesia period in comparison to the control (Fig. 3A, *$p<0.05$), while no significant changes were observed in the thalamus (Fig. 3B). At the protein level c-Fos was identified as the band at 65 kDa. Significant c-Fos decrease was noticed at 4 and 16 h after the treatment in the frontal cortex (Fig. 3C), while surprisingly strong decrease in the amount accompanied by slight decrease in the apparent molecular weight of c-Fos protein was detected in the thalamus during the 2-4 h post treatment period (Fig. 3D, *$p<0.05$ *vs*. control).

3.4. The effect of propofol anesthesia on intracellular localization of c-Fos

Since Western blot analysis revealed a decrease in c-Fos expression at 4 h after the treatment in both examined brain structures, we investigated how this change are coupled with intracellular localization of c-Fos (Fig. 4). In representative brain sections from control animals (Figs. 4A and 4B, the posterior cingulated/retrosplenial cortex and anterior thalamic nuclei, respectively) we observed co-localization of c-Fos with the neuronal marker, NeuN, in both nucleus and cytoplasm. Interestingly, in representative brain sections from propofol-exposed pups (Figs. 4C and 4D)

weaker nuclear c-Fos immunostaining was detected, especially in the thalamus (Fig. 4D). Such observation indicated that decreased c-Fos expression at 4 h after propofol treatment, revealed by Western blot analysis, could due to decreased expression of nuclear portion of c-Fos protein.

3.5. The effect of propofol anesthesia on the level of synaptophysin mRNA and protein expression

Synaptophysin is an abundant synaptic vesicle protein widely used as a marker for presynaptic terminals. Our experiments revealed that short-term propofol anesthesia down-regulates synaptophysin mRNA level in the frontal cortex of P7 rats during 2-4 h after the treatment (Fig. 5A, *$p<0.05$ *vs*. control), while no significant changes were observed in the thalamus (Fig. 5B). Western blot analyses revealed the protein level of synaptophysin significantly increased in the frontal cortex (50-75%) at 1, 8, 16 and 24 h after the treatment compared to the control group (Figs. 5C, *$p<0.05$) while in the thalamus it was significantly decreased (50%) during the 2-4 h post treatment period (Fig. 5D, *$p<0.05$).

3.6. The effect of propofol anesthesia on the level of GAP-43 and drebrin expression

Bearing in mind that BDNF plays a fundamental role in activity-dependent plasticity during development, we investigated by Western blot analysis the influence of the propofol treatment on the relative levels of GAP-43 (43 kDa), and drebrin (120 kDa), in the frontal cortex and in the thalamus of PND7 rats (Fig. 6).

In the frontal cortex of PND7 rats, the level of GAP-43 was significantly increased (25-30%) at 1, 8, 16 and 24 h after the treatment compared to the control group (Figs. 6A; *$p<0.05$). No significant changes in the levels of drebrin expression were detected in the frontal cortex of P7 rats (Fig. 6C).

In the thalamus of P7 rats the level of GAP-43 was unchanged (Fig. 6B), while drebrin expression was significantly increased specifically during 2-4 h post-anesthesia period (25-50%) and slightly decreased (22 %) at 24 h after the treatment (Fig. 6D; *$p<0.05$ *vs*. control).

3.7. The effect of neonatal propofol anesthesia on d-amphetamine-induced locomotor activity in adolescent and adult rats

The locomotor activity of adolescent P35 rats after saline and d-amphetamine injection is presented on Figs. 7A and 7B, respectively. After saline injection no significant differences in the activity of control and propofol pretreated rats was observed; statistical analysis revealed significant decrease in locomotor activity during the time (Fig. 7A, *p<0.05 vs. the first 30 min, $p<0.05 vs. the second 30 min). After d-amphetamine treatment the animals neonatally exposed to propofol showed important differences in locomotor activity in comparison to the control animals (Fig. 7B). Statistical analysis pointed to significant influence of propofol pretreatment ($F(1, 10) = 9.057$, $p=0.013$) and the time (($F(1, 30) = 15.939$, $p=0.001$). Namely, after d-amphetamine treatment propofol pretreated animals were significantly more active than control adolescent animals during the second and third 30 min of registration period (Fig. 7B, #p<0.05 vs. the same time point of control group). In contrast to the control group, significant decrease in d-amphetamine induced locomotor activity in propofol pretreated animals was observed only during the last 30 min period (Fig. 7B, *p<0.05 vs. the first 30 min, $p<0.05 vs. the second 30 min).

Animals neonatally exposed to propofol did not show any difference in the locomotor activity in comparison to the control group when tested as adults, either after saline injection (Fig. 7C) or after d-amphetamine injection (Fig.

7D). In both conditions significant decrease in the amount of locomotor activity was detected during the second hour of registration period (Figs. 7C and 7D, *$p<0.05$ vs. the first 30 min, \$$p<0.05$ vs. the second 30 min).

Before registration the rats were habituated to the experimental cages for 40 min. This activity was monitored as well but no significant differences were observed in spontaneous locomotor activity between control animals and those neonatally exposed to propofol anesthesia (data not shown).

4. DISCUSSION

In this study we presented *in vivo* evidence that a single-dose of the general anesthetic propofol applied to P7 rats induces complex brain region- and time-specific changes in the expression of BDNF and c-Fos. The changes were accompanied by alterations in the expression of synaptic plasticity markers as well. Moreover, the animals neonatally exposed to propofol showed transient increase in d-amphetamine induced locomotor response during adolescence. Together with our previous report that pointed to the short-term propofol anesthesia-induced neurodegeneration in P7 rat brain (Pesic et al., 2009), our present study fulfilled the knowledge about immediate changes in activity-regulated

processes in neonatal brain during post anesthesia period. This shed additional light on affected functional integrity/maturation of neural substrates that underlie stimulant drug responding.

There is no doubt that propofol anesthesia decreases neuronal activity in the cortex and thalamus, inducing reversible loss of consciousness (Franks, 2008), but the cellular consequences of such anesthesia action are largely unknown. Our study addressed that neonatal exposure to short-term propofol anesthesia decreased the expression of BDNF transcripts IV and VI (contributing to decrease in the expression of total BDNF mRNA) and c-Fos mRNA in the frontal cortex up to 24 hours after the treatment, while in the thalamus decreased expression of BDNF mRNAs was detected without changes in c-Fos mRNA. It has been widely accepted that BDNF and c-Fos mRNA expression is controlled by a neural activity accompanying Ca^{2+} influx into neurons (Tabuchi, 2008). Accordingly, our results indicate that in neonatal brain propofol anesthesia induced complex temporal and brain-region specific decline in Ca^{2+}-regulated signaling pathways required for activity-regulated gene expression, accentuating specificity of changes toward transcription during the post-anesthesia period. The findings are generally in agreement with *in vitro* obtained data about molecular mechanisms of propofol action (Kozinn et al., 2006; Martella et al., 2005). Moreover, up-

regulation of dopaminergic transmission that has been shown to mediate the emergence from propofol general anesthesia (Solt et al. 2014) could also contribute to the observed BDNF mRNA profile (Fumagalli et al., 2003).

After comparing the time-dependent changes in the levels of BDNF mRNA/protein in the same structure, we observed temporal and regional differences as an indication that following propofol anesthesia the activity of enzymes involved in mature BDNF protein production and/or BDNF transport could be affected (Head et al., 2009). The discrepancy between BDNF mRNA and protein has been observed in several treatments that modify neuronal activity (Elmer et al., 1998; Tropea et al., 2001). This phenomenon is also observed after repeated cocaine administration confirming that BDNF expression is controlled in a complex and highly dynamic fashion at the transcriptional and translational level, mRNA targeting as well as processing and secretion of the protein variants (Fumagalli et al., 2007). Recent findings indicate that different BDNF splice variants have a different translatability, contributing to the final amounts of the BDNF protein produced in response to the drugs (Vaghi et al., 2014). To the best of our knowledge comparative analysis of BDNF mRNA/protein expression after propofol exposure has not been reported, although some studies pointed to decreased expression of BDNF mRNA (Karen et al., 2013) and increased expression of BDNF

protein (Ponten et al., 2011) in the cortex of neonatal animals exposed to propofol. Moreover, general anesthesia with midazolam, isoflurane and nitrous oxide also leaded to an increase in BDNF in the cortex, and its decrease in the thalamus (Lu et al., 2006). Altogether these results suggest that in the neonatal brain, the level of BDNF changes in a brain region-specific manner in response to anesthesia exposure in general, rather than to propofol anesthesia *per se*, which remained to be deliberately examined. We also showed that propofol anesthesia did not influence the amount of total TrkB receptors in the frontal cortex and thalamus of P7 rats, but that it modulated TrkB phosphorylation (activity) by influencing extracellular BDNF levels. Through TrkB receptors endogenous BDNF acts as a target-derived and instructive messenger for long-lasting potentiation of $GABA_A$ receptor-mediated synaptic transmission, contributing to the activity-dependent developmental refinement of inhibitory synaptic networks (Kuczewski et al., 2008). Considering the importance of trophic support for multiple aspects of neuronal development and function our previous (Pesic et al., 2009) and current results indicate that during post-anesthesia period the frontal cortex had fewer disturbances than the thalamus, which undergo deficiency in both BDNF and NGF.

Inhibitory effect of propofol on stimulated c-Fos expression was previously reported, pointing to transcription-dependent mechanism that underlie anesthetic interference with synaptic plasticity related to amnesic properties of intravenous anesthetics (Fibuch and Wang, 2007; Kozin et al., 2006). Our study revealed that in neonatal brain short-term propofol anesthesia at time-dependent and structure specific manner significantly decreased basal expression of c-Fos during post-anesthesia period, demonstrating that in the thalamus this phenomenon could be related to augmented degradation of c-Fos protein as mRNA has been shown to be stable in this brain structure during the examined phase. The appearance of c-Fos as a 65 kDa band in both examined brain regions is an indication that the protein is highly phosphorylated and thus stable in developing brain, which is of importance considering its complex biological role. Namely, c-Fos is a part of the activator protein 1 (AP-1) transcription factor complex but recent findings indicate that it also has a non-genomic role in the cytoplasmic regulation of lipid synthesis required for membrane biogenesis (Caputto et al., 2014). Immunostaining revealed that observed sharp decrease in c-Fos level in the thalamus 4 hours after the treatment was greatly due to reduced c-Fos like immunoreactivity in the nucleus, pointing that propofol anesthesia in the neonatal brain transiently affected mainly genomic activity of c-Fos by decreasing the amount of available protein.

It has been proposed that c-Fos is, as a part of multifaceted set of signaling molecules, participated in the triggering of the expression of genes important for neuronal plasticity, including BDNF (Dong et al., 2006). In agreement with this hypothesis our study showed that the time window of decreased c-Fos level in neonatal brain after short-term propofol anesthesia exposure matched exon IV- and exon VI-containing BDNF mRNAs decrease particularly in the thalamus. Although there are indications that BDNF can have an important role in the regulation of c-Fos expression (Cohen et al., 2011), our results indicate that in examined conditions increased BDNF/phospho-TrkB level is not crucial for the maintenance of stable c-Fos mRNA level in the frontal cortex.

An important feature of the developing brain is its remarkable plasticity in response to the experience. Our study revealed that short-term exposure to propofol anesthesia induced time- and brain region-specific changes in synaptophysin expression that was used as a specific presynaptic marker. Discrepancy between mRNA and protein level within examined structures appeared, which was not unexpected considering general thought that the bulk of synaptic proteins is synthesized in the cell body and thereafter transported to synaptic sites. Accordingly, impressive similarity between decreased synaptophysin mRNA expression in the frontal cortex and decreased protein level in the

thalamus was observed. Moreover, 30 kDa synaptophysin fragment, which was described as a breakdown product of calpain-1 activity (Lee et al., 2008), appeared along with the decrease in synaptophysin band (38 kDa) indicating that increased Ca^{2+}-dependent degradation contributed to the observed result (Wu and Lynch, 2006; Milanovic et al., 2010). Considering the role of synaptophysin in presynaptic functions including endocytosis of synaptic vesicles (Kwon and Chapman, 2011), present findings suggest that transient and time-specific decrease of functional presynaptic activity is induced in the thalamus of neonatal brain after short-term propofol exposure. Consequently, simultaneous increase in the expression of drebrin indicates that the non-functional synaptic contacts in the thalamus of P7 rats after short-term propofol anesthesia might appear (Mizui et al., 2005). On the other side, increased GAP-43 level was detected specifically in the cortex, which along with increased synaptophysin level in the same brain structure indicates that increase in synaptogenesis could have occurred. We presume that elevated BDNF/phospho-TrkB level at least in part contributed to this phenomenon, in view of recently described relationship between BDNF induced axon branching and synaptic vesicle cycling (Granseth et al., 2013).

Potential functional consequences of early anesthesia exposure were tested in adolescent and adult animals exposed to psychostimulant drug d-amphetamine, by assessing locomotor hyperactivity. Obtained results pointed to increased, time-related hyperlocomotion of adolescent rats neonatally treated with propofol. Briefly, d-amphetamine induced motor action is controlled by the cortical inhibition of striatal dopaminergic activity, with the important modulatory role of cortical GABAergic system (Karler et al., 1998). Moreover, recent data strongly suggest that amphetamine-stimulated monoamine release in the primary motor cortex and hyperlocomotor activity depend on the integrity of thalamocortical signaling (Mabrouk et al., 2014). Accordingly, the results observed in our study indicate that disturbed cortical control over subcortical structures and/or affected thalamo-cortical signaling in animals neonatally exposed to propofol might occur later during development. This presumption is in agreement with the facts that decreased BDNF gene expression specifically influences development and function of GABAergic synapses in the cortex (Hong et al., 2008) and that prolonged hyperlocomotion following acute amphetamine exposure was detected in *Bdnf*$^{+/-}$ mice (Saylor and McGinty, 2008). Affected ability of propofol exposed animals to control stimulated dopamine activity, which is primarily terminated by re-uptake into the presynaptic neuron terminals (Norregaard and

Gether, 2001), could not be neglected. Neurophysiologically, a depression of thalamo-cortical activity was reported (Franks, 2008), although convincing evidences suggest that clinical effects of propofol are due to disconnection of functional connectivity over cortico-cortical and cortico-subcortical networks, i.e. cortico-thalamic (Velly et al., 2007) and cortico-striatal networks (Mhuircheartaigh et al., 2010). Thus, neonatal exposure to propofol could be viewed as an early challenge for the cortico-striato-thalamo-cortical loops that are involved in different behaviors, including drug-induced and naturally occurring psychoses that mainly emerge during adolescence (Paus et al., 2008). Different behavioral responses to diazepam in rats that were neonatally exposed to propofol anesthesia were recently reported (Ponten et al., 2011) and our present study additionally pointed that the long-term outcome of neonatal exposure to anesthesia could be viewed through the response to psychotropic medications during adolescence, the developmental period characterized by widespread adaptations in brain anatomy and functioning.

Methodological limitations of the study should be noted. We examined the effects of propofol manufactured for human use (Recofol®), i.e. the effects of propofol and its solvent. Although currently marketed propofol formulation has a number of undesirable properties that are largely due to the lipid emulsion, it seems that reformulation of propofol

can alter its pharmacokinetic and pharmacodynamic characteristics (Egan, 2010). Others who have investigated propofol in neonatal rats used physiological saline (Fredriksson et al., 2007; De Roo et al., 2009; Milanovic et al., 2010) or lipid emulsion (Nakao et al., 2003; Oscarsson et al., 2001; Turina et al., 2008; Vutskits et al., 2005) as a vehicle, showing that the vehicle alone does not produce any effect considering actin polymerization, c-Fos expression, neurite retraction, dendritic spine density, dendritic morphology, neurodegeneration and behavioral deficit. Because of all of the above-mentioned facts, we did not examine the effects of the lipid emulsion separately. However, we cannot exclude the possibility that observed propofol anesthesia-induced effects are at least in part due to the intralipid action. Limitation of the study could be also the usage of whole cell extract for the analysis of proteins with different subcellular distribution, without doing the correct subfractionation.

4.1. Conclusion

This study explains how activity-regulated events within different cellular compartments (cytoplasmic or nuclear) and through specific synaptic elements (pre- or postsynaptic), are integrated into region specific, immediate

response of neonatal brain to propofol anesthesia. Moreover, neonatal exposure to propofol appeared to be an early challenge for neural substrates that underlie stimulant drug responding, resulting in temporary altered response to acute psychotropic stimulation during adolescence. Obtained results shed a new light on consequences of exposure to general anesthesia during brain development and its safety in pediatric medicine. Additional experiments should be performed to better understand biochemical changes induced by early anesthesia exposure and their functional outcome. In that sense age-related molecular characterization of animals neonatally exposed to general anesthesia is of importance, as well as their behavioral reaction to different experimental paradigms including a challenge with spectrum of psychoactive drugs.

Acknowledgements

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Conflict of interest statement

No conflict of interest to declare.

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TABLE I. Primer Sequences and Annealing Temperature Used for Semiquantative RT-PCR Analyses

Gene	Primers (5'–3')	Size (bp)	PCR profile	No. of cycles
Exon IV BDNF	CTCTGCCTAGATCAAATGGAGCTTC GAAGTGTACAAGTCCGCGTCCTTA	553	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	32
Exon VI BDNF	GCTGGCTGTCGCACGGTCCCCATT GAAGTGTACAAGTCCGCGTCCTTA	625	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	32
Exon IX BDNF	CACTCCGACCCCGCCCGCCG TCCACTATCTTCCCCTTTTA	364	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	26
c-Fos	GACCGAGATTGCCAATCTAC GGAAACAAGAAGTCATCAAAGG	285	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	35
β-actin	TGGACATCCGCAAAGACCTGTAC TCAGGAGGAGCAATGATCTTGA	142	Depends on target gene	
GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	306	Depends on target gene	

FIGURE LEGENDS

Figure 1. Propofol treatment down-regulates the level of BDNF mRNA in P7 rat brain.

RT-PCR analysis is used to examine the effect of propofol anesthesia (25 mg/kg) on the expression of exon IV- and exon VI-containing BDNF mRNAs, and on the level of total BDNF mRNAs in the frontal cortex (A, C and E) and thalamus (B, D and F) of P7 rats. Each graph is accompanied by a representative gel. The data were expressed as percentage to the control (0 h) and are represented as mean ± SEM (n=4; each in triplicate). The values obtained at 0 h were defined as 100%. Asterisk indicate significant difference from the control according to the Kruskal-Wallis ANOVA followed by the U post-hoc test (*P<0.05).

Figure 2. Propofol treatment affects the levels of BDNF and phosphorylated TrkB protein in P7 rat brain.

Western blot analysis is used to examine the effect of propofol anesthesia (25 mg/kg) on BDNF, total TrkB and phosphorylated TrkB protein expression in the frontal cortex (A, C) and thalamus (B, D) of P7 rats. Each graph is accompanied by a representative immunoblot. The data were expressed as percentage to the control (0 h) and are represented as mean ± SEM (n=4; each in triplicate). The values obtained at 0 h were defined as 100%. Asterisk indicate significant difference from the control according to the Kruskal-Wallis ANOVA followed by the U post-hoc test (*P<0.05).

Figure 3. Propofol treatment affects c-Fos expression in P7 rat brain.

RT-PCR and Western blot analyses are used to determine the effect of propofol anesthesia (25 mg/kg) on the expression of c-Fos mRNA and protein in the frontal cortex (A and C, respectively) and thalamus (B and D, respectively). Each graph is accompanied by a representative gel or immunoblot. The data were expressed as percentage to the control (0 h) and are represented as mean ± SEM (n=4; each in triplicate). The values obtained at 0 h were defined as 100%. Asterisk indicate significant difference from the control according to the Kruskal-Wallis ANOVA followed by the U post-hoc test (*$P<0.05$).

Figure 4. The expression of c-Fos in the cortex and thalamus of P7 rat brain.

The expression of c-Fos was detected in neurons (labeled by Neu-N) of the posterior cingulated/retrosplenial cortex (A-H) and anterior thalamic nuclei (I-P) of control animals (A-D and I-L) and in those exposed to propofol anesthesia (E-H and M-P). Nuclear localization was confirmed by DAPI staining. Weaker nuclear c-Fos immunostaining could be observed in propofol exposed animals, 4 h after the treatment. Scale bar 100 μm.

Figure 5. Propofol treatment affects synaptophysin expression in P7 rat brain.

Real-time RT-PCR and Western blot analyses are used to determine the effect of propofol anesthesia (25 mg/kg) on the expression of synaptophysin mRNA and protein in the frontal cortex (A and C, respectively) and thalamus (B and D, respectively). The data were expressed as percentage to the control (0 h) and are represented as mean ± SEM (n=4; each in triplicate). The values obtained at 0 h were defined as 100%. Asterisk indicate significant difference from the control according to the Kruskal-Wallis ANOVA followed by the U post-hoc test (*P<0.05).

Figure 6. Propofol treatment affects GAP-43 and drebrin expression in P7 rat brain.

Western blot analyses are used to determine the effect of propofol anesthesia (25 mg/kg) on the protein expression in the frontal cortex (A and C, respectively) and thalamus (B and D, respectively). The data were expressed as percentage to the control (0 h) and are represented as mean ± SEM (n=4; each in triplicate). The values obtained at 0 h were defined as 100%. Each graph is accompanied by a representative gel or immunoblot. Asterisk indicate significant difference from the control according to the Kruskal-Wallis ANOVA followed by the U post-hoc test (*P<0.05).

Figure 7. Neonatal propofol anesthesia affects hyperlocomotor response to d-amphetamine in adolescent rats.

At P7 the animals were injected either propofol (25 mg/kg) or saline (0.9% NaCl, 1 ml/kg) and allowed to recover. Monitoring of locomotor activity was performed on adolescent (P35) and adult (P90) rats, after the injection of either saline (1 ml/kg; Figs. 7A and 7C) or d-amphetamine (1.5 mg/kg/ml, Figs. 7B and 7D). Each animal was tested only once. Before registration, the rats were habituated to the experimental cages for 40 min. The data are expressed as total activities (mean±SEM, n=6) for 30 min periods. Significant difference according to the two-way ANOVA followed by the LSD post-hoc test: *$p<0.05$ compared to the activity of the same group during the first 30 min of registration period; \$$p<0.05$ compared to the activity of the same group during the second 30 min of registration period; #$p<0.05$ compared to the same time point of propofol-pretreated group.

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BDNF	GAAGTGTACAAGTCCGCGTCCTTA			
Exon IX	CACTCCGACCCCGCCCGCCG	364	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	26
BDNF	TCCACTATCTTCCCCTTTTA			
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	GGAAACAAGAAGTCATCAAAGG			

β-actin	TGGACATCCGCAAAGACCTGTAC	142	Depends on target gene
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Exon IX	CACTCCGACCCCGCCCGCCG	364	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	26
BDNF	TCCACTATCTTCCCCTTTTA			
c-Fos	GACCGAGATTGCCAATCTAC	285	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	35
	GGAAACAAGAAGTCATCAAAGG			
β-actin	TGGACATCCGCAAAGACCTGTAC	142	Depends on target gene	
	TCAGGAGGAGCAATGATCTTGA			
GAPDH	CGGAGTCAACGGATTTGGTCGTAT	306	Depends on target gene	

AGCCTTCTCCATGGTGGTGAAGAC

TABLE I. Primer Sequences and Annealing Temperature Used for Semiquantative RT-PCR Analyses

Gene	Primers (5'–3')	Size (bp)	PCR profile	No. of cycles
Exon IV BDNF	CTCTGCCTAGATCAAATGGAGCTTC GAAGTGTACAAGTCCGCGTCCTTA	553	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	32
Exon VI BDNF	GCTGGCTGTCGCACGGTCCCCATT GAAGTGTACAAGTCCGCGTCCTTA	625	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	32
Exon IX BDNF	CACTCCGACCCCGCCCGCCG TCCACTATCTTCCCCTTTTA	364	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	26
c-Fos	GACCGAGATTGCCAATCTAC GGAAACAAGAAGTCATCAAAGG	285	94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec;	35
β-actin	TGGACATCCGCAAAGACCTGTAC TCAGGAGGAGCAATGATCTTGA	142	Depends on target gene	
GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	306	Depends on target gene	

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GAPDH	CGGAGTCAACGGATTTGGTCGTAT	306	Depends on target gene
	AGCCTTCTCCATGGTGGTGAAGAC		