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ORIGINAL ARTICLE

Neonatal Propofol Anesthesia Changes Expression of Synaptic Plasticity Proteins and Increases Stereotypic and Anxyolitic Behavior in Adult Rats

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Abstract Propofol is a general anesthetic commonly used in pediatric clinical practices. Experimental findings demonstrate that anesthetics induce widespread apoptosis and cognitive decline in a developing brain. Although anesthesia-mediated neurotoxicity is the most prominent during intense period of synaptogenesis, the effects of an early anesthesia exposure on the synapses are not well understood. The aim of this study was to examine the effects of neonatal propofol anesthesia on the expression of key proteins that participate in synaptogenesis and synaptic plasticity and to evaluate long-term neurobehavioral abnormalities in the mature adult brain. Propofol-injected 7-day-old rats were maintained under 2-, 4-, and 6-h-long anesthesia and sacrificed 0, 4, 16, and 24 h after the termination of each exposure. We showed that propofol anesthesia strongly influenced spatiotemporal expression and/or proteolytic processing of crucial presynaptic (GAP-43, synaptophysin, α-synuclein), trans-synaptic (*N*-cadherin), and postsynaptic (drebrin, MAP-2) proteins in the cortex and thalamus. An overall decrease of synaptophysin, α-synuclein, *N*-cadherin, and drebrin indicated impaired function and structure of the synaptic contacts immediately after anesthesia cessation. GAP-43 and MAP-2 adult and juvenile isoforms are upregulated following anesthesia, suggesting compensatory mechanism in the maintaining of the structural integrity and stabilization of developing axons and dendritic arbors. Neonatal propofol exposure significantly altered spontaneous motor activity (increased stereotypic/repetitive movements) and changed emotional behavior (reduced anxiety-like response) in the adulthood, 6 months later. These findings suggest that propofol anesthesia is synaptotoxic in the developing brain, disturbing synaptic dynamics and producing neuroplastic changes permanently incorporated into existing networks with long-lasting functional consequences.

Keywords Developing brain · Propofol anesthesia · Synaptic plasticity · Behavior · Neurotoxicity

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Introduction

General anesthesia is commonly used in pediatric surgery of neonates and young children (Jevtovic-Todorovic et al. 2010). However, experimental findings have demonstrated that general anesthetics, including propofol (2,6-diisopropylphenol), induce developmental neurotoxicity and profound neuroapoptosis, thereby leading to behavioral and functional deficits in adult animals (Milanovic et al. 2016; Pesic et al. 2015; Milanovic et al. 2014; Ming et al. 2014; Yu et al. 2013; Karen et al. 2013; Pesic et al. 2009, Milanovic et al. 2010; Nikizad et al. 2007). Numerous preclinical data are supported by clinical epidemiological studies that suggest that children having more than one exposure to anesthesia before the age of 4 have behavioral disturbances and learning deficit later on in life (Wilder 2010; Vutskits 2012). Accordingly, early exposures to anesthesia may have life-long consequences on the CNS and are known to be risk factors for both neurodevelopmental and neurodegenerative diseases (Jevtovic-Todorovic et al. 2010).

Based on the currently available data, the most important determinant of the vulnerability of developing brain appears to be timing of the exposure to anesthesia relative to the time point in synaptogenesis (Rizzi et al. 2010). During the period of intense synaptogenesis, a highly programmed neuronal communication is of vital importance for survival and functioning of immature neurons, and their incorporation into neural networks. An essential parameter regulating synaptic connectivity, wiring pattern, and functional circuitry is neuronal activity (Miller and Kaplan 2003). General anesthetics, including propofol, induce suppression of neuronal activity by potentiating inhibitory neurotransmission through gamma-amino butyric acid (GABA) receptors and/or inhibiting excitatory glutamatergic neurotransmission through blockade of *N*-methyl D-aspartate (NMDA) receptors. Anesthesia-induced disturbances in the fine equilibrium between excitatory and inhibitory neurotransmission might lead to persistent disruption of developing or already established synaptic connections (Sitditkova et al. 2013; Yu et al. 2013; Pearn et al. 2012). Several experimental in vivo and in vitro studies demonstrated the effects of anesthetics on synaptogenesis, synaptic transmission, and synaptic plasticity during the brain growth spurt (Briner et al. 2011; Sanchez et al. 2011; Lunardi et al. 2015; De Roo et al. 2009). Morphometric analysis revealed loss of both inhibitory and excitatory synapses, altered presynaptic mitochondrial morphogenesis, and changes in the dendritic spine densities and morphology, dendritic length, and arborization pattern (Lunardi et al. 2015; Vutskits 2005). Inappropriate synapse formation or structure is thought to underlie a variety of neurodevelopmental disorders (Sprung et al. 2012; Briner et al. 2011). Also, a growing body of human pathological evidence indicates that many psychiatric and neurological disorders, ranging from mental retardation and autism to Alzheimer's disease and addiction, are accompanied by alternations in spine morphology and synapse number (Schaefers and Teuchert-Noodt 2013; Vutskits 2012). It is thus tempting to speculate that anesthetic-induced alterations in synaptic density and structure might stand as a morphological basis underlying impaired neurocognitive performance associated with early anesthesia (Vutskits 2012).

While a large amount of information has been accumulated on morphological changes, the molecular constituents of synapses and the regulatory mechanisms for the formation of synaptic junctions after general anesthesia are still poorly understood. The current study was undertaken to evaluate the effect of the early propofol anesthesia on the temporal and spatial expression of crucial proteins that take part in synaptic function and plasticity. Hence, we assessed the expression of presynaptic proteins growth-associated protein 43 (GAP-43), synaptophysin (SPH) and α-synuclein (α-SYN), transsynaptic protein *N*-cadherin (N-cad) and postsynaptic markers, microtubule-associated protein 2 (MAP-2), and drebrin in the immature brain following different-duration exposures to propofol anesthesia. This study clearly show that propofol anesthesia, applied at the peak of synaptogenesis, disturbed synaptic organization in the developing brain that had long-lasting functional consequences manifested through defective repetitive/stereotypic activity and anxyolitic behavioral response of adult animals, 6 months after a single propofol exposure.

Materials and Methods

Ethic Statement

All experimental procedures were in compliance with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research, University of Belgrade, and in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH).

Animals and Treatment

Seven-day-old (P7) male Wistar rats (average body weight 12–14 g) were used in experiments. The procedures were designed to minimize the suffering of the animals and the number of rats used. Rat pups were separated from their mothers and placed in a temperature-controlled incubator set to an ambient temperature of 35–36 °C during anesthesia and first hour of recovery periods, after which they were returned to dam to feed. The treatment was performed by administration of propofol manufactured for intravenous human use (Recofol®; Schering Oy, Turku, Finland). Loss of the righting reflex served as an indicator of anesthetic-induced unconsciousness and sleeping time. A dose of 20 mg/kg that impaired the righting reflex for 43 ± 5 min was used. During anesthesia procedure, the well-being of animals was closely monitored by observational methods. An indication of respiratory function and oxygenation was obtained by careful assessing of respiratory pattern (regular thoracic movement) and color of mucosa membrane and extremities (pups appeared pink, with no visible signs of cyanosis), respectively. Other studies, using similar anesthetic regimen, reported that prolonged propofol anesthesia did not alter the physiological parameters such as pO2, pCO2, lactate, and blood sugar concentrations in rats and mice (Yu et al. 2013; Bercker et al. 2009; De Roo et al. 2009). We did not examine the effect of the lipid emulsion as a vehicle in this study, which might be a limiting factor; however, it was shown that the lipid vehicle did not change different biochemical markers in vivo and in vitro (Li et al. 2016; Turina et al. 2008; Vutskits et al. 2005).

Experimental Procedures

To prevent possible litter effects, a single pup from a litter was assigned to every single group of experimental rats mentioned at the appropriate points in this article. P7 rat pups were administered either 2, 4, or 6 bolus injections of 20 mg/kg of propofol intraperitoneally at 1 h intervals in order to achieve either 2-, 4-, or 6-h-long anesthesia (Milanovic et al. 2010). The animals (n = 4 per time point) were decapitated either immediately after cessation of the exposure times (designated as the 0 h time point) or after the recovery periods that lasted 4, 16, or 24 h following termination of propofol exposure (and are referred to as 4, 16, and 24 h time points, respectively) (Fig. 1). Control animals, which were separated from their mothers, received 2, 4, or 6 injections of physiological saline at the same intervals as propofol-treated littermates and were sacrificed at 0 h time point. Whole brains were isolated (n = 3 per group) for use in histological studies.

Tissue Extracts and Western Blot Analysis

To obtain whole-cell extracts, the tissue was homogenized with a Dounce homogenizer in 10 vol (w/v) of lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA pH 8.0, 1 mM EGTA, pH 7.2, 0.5% Triton X-100) that contained a complete cocktail of phosphatase and protease inhibitors (Roche, Mannheim, Germany). The homogenates were sonicated and centrifuged at 16000g at 4 °C for 30 min. The supernatants were collected and stored at −70 °C until use. Protein concentrations were determined by the bicinchonic acid micro-protein assay (Micro BCA Protein Assay Kit; Pierce Inc., Rockford, IL, USA) with albumin as a standard.

Proteins (10–20 μg per lane) were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Bioscience, Otelfingen, Switzerland). The membranes were blocked at room temperature for 1 h in 3% bovine serum albumin in Tris-buffered saline/0.1% Tween 20 (TBS-T), followed by incubation for 2 h or overnight with primary antibodies: anti-GAP-43 (Millipore), anti-SPH (gift from Dr. Jahn Reinhard, Max Planck Institute, Gottingen, Germany), anti-α-SYN (Abcam), N-Cad (Transduction Lab), anti-drebrin (Abcam), anti-MAP-2(Sigma) that recognize MAP-2 a/b and c isoforms, and anti-MAP-2 (Millipore) that recognize MAP-2a/b isoforms. The immunoblots were processed with horseradish peroxidase-conjugated anti-rabbit (sc-2350, Santa Cruz) or anti-mouse antibodies (PO160, Dako) in TBS-T for 1 h at room temperature. Three washes with 0.3% Tween 20 in Tris-buffered saline were performed between all steps. All blots were incubated with anti-β-actin or anti-GAPDH antibodies to correct for any differences in protein loading. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham Bioscience) after exposure on X-ray film. All films were densitometrically analyzed using the computerized image analysis program ImageQuant 5.0.

Motor Activity Testing in the Open Field

Behavioral experiments were performed between 09:00 hours and 14:00 hours. Six-month-old animals neonatally exposed to propofol anesthesia (n = 6 per group) and control, saline-treated animals (n = 6 per group), were first tested in the open field and then in the light/dark box,

Motor activity testing lasted for 3 consecutive days and was performed to assess habituation ability of the animals (Milanovic et al. 2016). During each day, monitoring of motor (exploratory) activity started immediately after the exposure of animals to Opto-Varimex cages (Columbus Instruments, OH) that were linked on-line to an IBM-PC-compatible computer and lasted for 30 min. Individual monitoring for each animal was done. 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. Cages were placed in a light- and sound-attenuated room provided with indirect and homogenous illumination (150 lx in the centre of the open field arena). Data were analyzed using the 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. Behavioral variables were locomotor activity, repetitive/stereotypic-like movements, and vertical activity. Locomotor activity was defined as a trespass of three consecutive photo-beams. The number of repetitive/

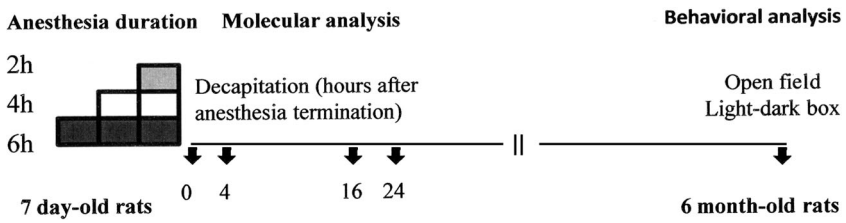

Fig. 1 Timeline of the experiment. Seven-day-old animals were sacrificed immediately after termination of 2-, 4-, and 6-h propofol anesthesia (0 h time point) or at different post exposure intervals (4, 16 and 24 h). A group of animals that were exposed to 6-h-long propofol anesthesia was behaviorally tested 6 months later

stereotypic-like movements was defined as the number of repeated breaks of the same beam in 1/10th of a second. Vertical activity was measured by recording the number of beams that were broken by rearing of the animal. After testing, subjects were removed from the activity box and returned to their home cage in colony room. The apparatus was cleaned with 70% ethanol after each use and allowed to dry before the next subject was tested.

Light/Dark Box Testing

Behavior of the animals in the light/dark box was examined 5 days after habituation testing. The light/dark box was made of white and black opaque Plexiglas (31 × 31 × 36 cm light compartment, 20 × 31 × 36 cm dark compartment). The chambers were connected by a 10 × 10 cm door in the middle of the wall separating the two chambers. Animals were placed in the middle of the light chamber facing a side away from the door and then released. Behavior was recorded by video camera for 10 min. After testing, subjects were removed from the light/dark box and returned to their home cage in colony room. The apparatus was cleaned with 70% ethanol after each use and allowed to dry before the next subject was tested.

After the testing the quantitative evaluation of the animal behavior in the light/dark box test was performed by watching the recorded material. Behavioral variables analyzed included the duration of time spent in the light chamber, the duration of time spent in the dark chamber, the frequency of stretches from the dark chamber into the light chamber (not all 4 ft in the light chamber), and the duration of time spent in the door area during stretching.

Statistical Analysis

Semi-quantitative evaluation of protein levels detected by Western immunoblotting was performed by densitometric scanning using the computerized image analysis program ImageQuant 5.0. The data are presented as percentages (means ± SEM) relative to the control samples assumed to be 100% (depicted by a black line on the graphs). Differences between the experimental groups were tested using Kruskal-Wallis nonparametric ANOVA with Mann-Whitney's post hoc *U* test. Significance was reported at $p < 0.05$.

Statistical analyses of behavioral data were performed using the Statistica 6.0. Graphical presentation of results is given. The data obtained for the habituation sessions were presented as the summary of locomotor, stereotypy-like and vertical activities for the whole 30 min registration period. The data obtained in the light/dark box test were given for 10 min session. Normality of the data was estimated by Shapiro-Wilk test. All analyzed behavioral variables reached the criterion for normal distribution, except vertical activity data which were therefore sqrt transformed. The data obtained for the habituation sessions were analyzed by a two-way analysis of variance (ANOVA) with propofol pretreatment and time (repeated measure) as factors. Subsequent comparisons were made using the Fisher LSD test. Data obtained in the light/dark box testing, as normal distribution was reached, were analyzed by *t* test for independent samples, with propofol pretreatment as a factor.

Results

Propofol Anesthesia Increases Full-Length and Cleaved GAP-43 Protein Levels in the Cortex and Thalamus of P7 Rats

To determine the effect of propofol anesthesia on the major markers of synaptic plasticity in immature rat brain, P7 pups were exposed to propofol anesthesia for 2, 4, or 6 h and were sacrificed at two earlier (0 and 4 h) and two later (16 and 24 h) time points following termination of each exposure.

We intended to examine expressions of intact and cleaved GAP-43 protein, which is very abundant in neuronal growth cones during development, in the cortex and thalamus by Western blot analysis. The data showed that expression of full-length GAP-43 protein (43 kDa) was significantly increased (up to 100%) in similar manner in the cortex (Fig. 2a) and thalamus (Fig. 2b) after all three propofol exposures.

Evaluation of cleaved GAP-43 (~37 kDa) level that is generated as a product of calpain-mediated proteolytic degradation, pointed to notable differences in-between two structures as basal level of this fragment is undetectable in the cortex. However, propofol induced a robust increase of GAP-43 proteolysis during the first 4 h after cessation anesthesia in both structures (Fig. 2a, b, representative Western blots; quantification data not shown). Thereafter, the level of GAP-43 fragment had a steep decline but remained elevated until 24 h post exposure.

Propofol Differentially Regulates Expression of SPH in the Cortex and Thalamus of P7 Rats

Synaptophysin (38 kDa) is a protein abundantly present in the membranes of neurotransmitter containing vesicles in presynaptic terminals. Western blot analysis showed that propofol anesthesia affected expression of SPH in the cortex and thalamus differently (Fig. 3a, b). In the cortex, there was strong decrease in SPH protein levels (around 30% of control value immediately after 2 h exposure) that remained reduced during 16 h after 2- and 4-h-long anesthesia (Fig. 3a). A 6-h-long exposure induced remarkable but transient decrease of SPH level (~50% under control,

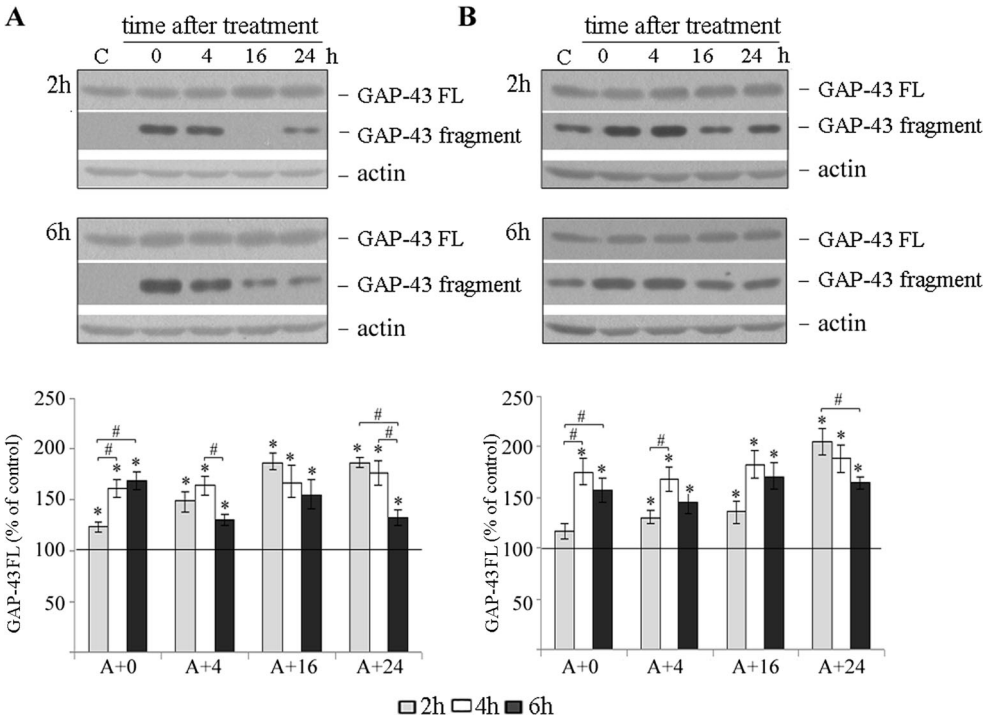

Fig. 2 Time course of GAP-43 protein expression in the developing brain after exposure to propofol anesthesia. The levels of GAP-43 expression were assessed by Western blot analysis of total protein extracts obtained from the cortex (**a**) and the thalamus (**b**) of P7 rats. The results are presented for animals at different recovery time points (0, 4, 16, and 24 h) after exposures to propofol anesthesia for 2, 4, and 6 h. Representative Western blots are shown with protein bands corresponding to full-length GAP-43 (GAP-43 FL), GAP-43 proteolytic fragment, and β-actin, which served as an internal control of the protein load. *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. other exposures at the same time point

$p < 0.05$) at 4 h time point. In the thalamus (Fig. 3b), elevated SPH expressions were observed after all three exposures to propofol, reaching a peak 24 h after termination of anesthesia (rise by50–100% compared to control). The only significant but transient decrease of SPH in thalamus was induced by a 2-h-long exposure to propofol (50% of control value at 0 h time point, $p < 0.05$).

Propofol Treatment Changes Expression of α-SYN in the Cortex and Thalamus of P7 Rats

Alpha-SYN is a small (18 kDa) protein richly expressed in presynaptic terminals in close association with membrane of synaptic vesicles. Western blot analysis revealed that propofol anesthesia was capable of changing α-SYN protein

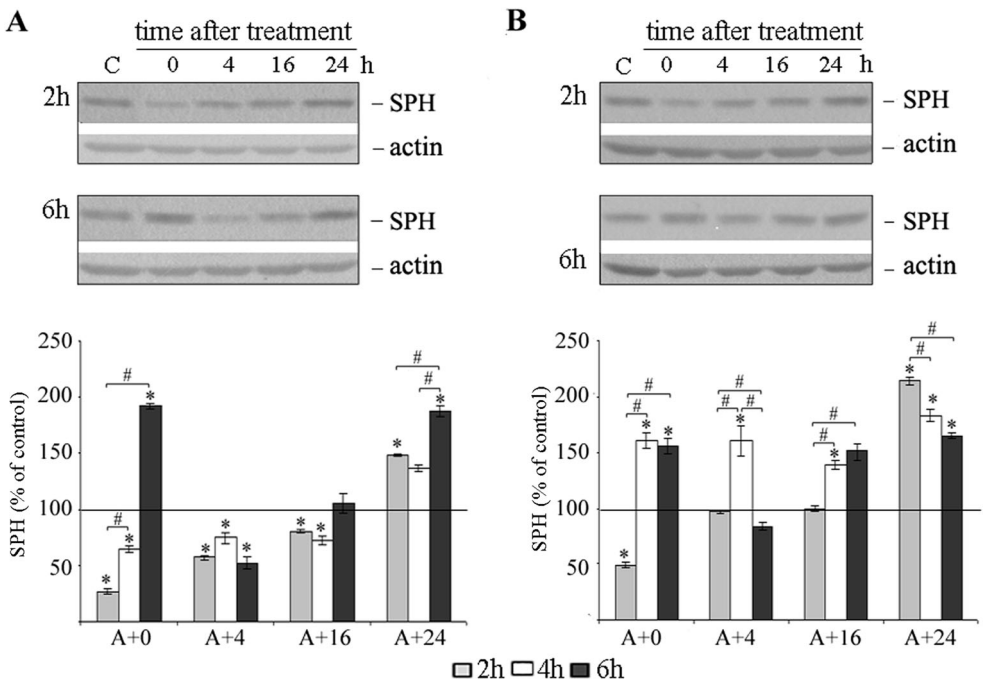

Fig. 3 Time course of SPH protein expression in the developing brain after exposure to propofol anesthesia. The levels of SPH expression were assessed by Western blot analysis of total protein extracts obtained from the cortex (**a**) and the thalamus (**b**) of P7 rats. The results are presented for animals at different recovery time points (0, 4, 16, and 24 h) after exposures to propofol anesthesia for 2, 4, and 6 h. Representative Western blots are shown with protein bands corresponding to SPH and β-actin, which served as an internal control of the protein load. *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. other exposures at the same time point

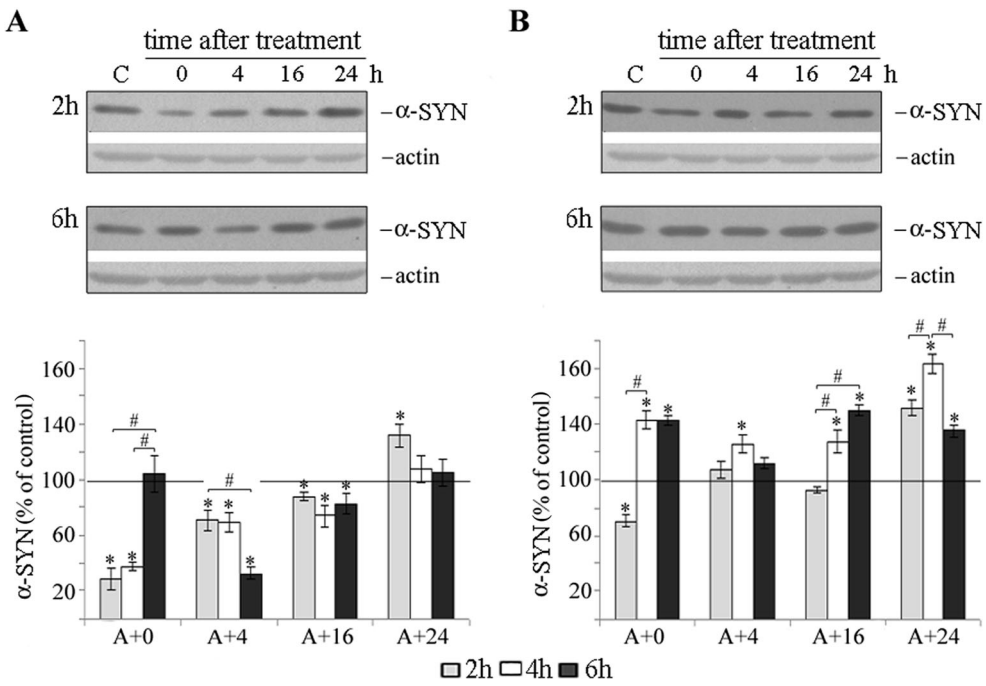

Fig. 4 Time course of α-SYN protein expression in the developing brain after exposure to propofol anesthesia. The levels of α-SYN expression were assessed by Western blot analysis of total protein extracts obtained from the cortex (**a**) and the thalamus (**b**) of P7 rats. The results are presented for animals at different recovery time points (0, 4, 16, and 24 h) after exposures to propofol anesthesia for 2, 4, and 6 h. Representative Western blots are shown with protein bands corresponding to α-SYN and β-actin, which served as an internal control of the protein load. *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. other exposures at the same time point

expression, regardless on duration of exposure (Fig. 4a, b). In the cortex, there was a robust decrease of α-SYN levels (up to 70%, $p < 0.05$) at first 4 h after anesthesia termination. However, 24 h after termination of anesthesia, α-SYN returned to control level or slightly above it (38% after 2-h-long anesthesia, $p < 0.05$). In the thalamus, on the contrary, there was a general increase of α-SYN expression around 40–60% following all three exposures. A significant decrease was measured only immediately following 2 h exposure as revealed by ~30% fall compared to control value (Fig. 4b, A + 0 time point).

Propofol Changes Expression of *N*-Cadherin in the Cortex and Thalamus of P7 Rat Brains

Considering that propofol anesthesia changed significantly levels of three presynaptic markers, we next sought to examine if the expression of the well-known trans-synaptic adhesive protein N-cad was also affected by exposure to propofol. Western blot analysis showed significant effect of propofol on the levels of this protein in both the cortex and thalamus (Fig. 5a, b). In the cortex, there was robust decrease (by 60–70% of the control value) of ProN-cad (135 kDa) immediately after termination of all three propofol treatments (Fig. 5c). This was followed by recovery and rise up to 40%, ($p < 0.05$) above the control value 24 h after anesthesia cessation. In contrast, expression of ProN-cad in the thalamus was many-fold increased throughout entire time period examined (Fig. 5d). The most prominent rise, almost 4-fold, was measured after 4 h propofol exposure.

In parallel, propofol treatment changed levels of 90 kDa mature N-cad that originates from ProN-cad after convertases-mediated removing of prodomain. In the cortex, there were no changes in the level of 90 kDa N-CAD following termination of propofol exposures (Fig. 5e). However, 4 h after, there was a drop of cleaved N-cad to 20–45% of the control level. A further decrease was registered throughout 24 h time period reaching the minimal value at the end of treatment when 10–20% of the control valued was measured. In the thalamus (Fig. 5f), there was decrease in N-cad cleavage (30 and 63% of the control value, respectively) immediately after termination of 2 and 4 h exposures, with no change for 6 h treatment. Throughout 24 h time period, a decline of cleaved N-cad was measured after all three propofol exposures, being most prominent following 4 h propofol anesthesia (~10% of control level, $p < 0.05$).

Time Course of Drebrin Protein Levels in the Cortex and Thalamus of P7 Rat Brain Following a 2-, 4-, and 6-h-Long Propofol Treatment

Propofol anesthesia was shown to induce marked changes of key pre- and trans-synaptic proteins. Therefore, we next examined expression of two proteins important for postsynaptic structure and function, drebrin, and MAP-2.

Drebrin (120 kDa) is postsynaptic protein particularly concentrated in spines. Expression of drebrin was significantly influenced by propofol anesthesia in both brain structures examined (Fig. 6a, b). In the cortex, there was a reduction of drebrin levels by 30–50% immediately after termination of all three exposures to propofol anesthesia (Fig. 6a). This was

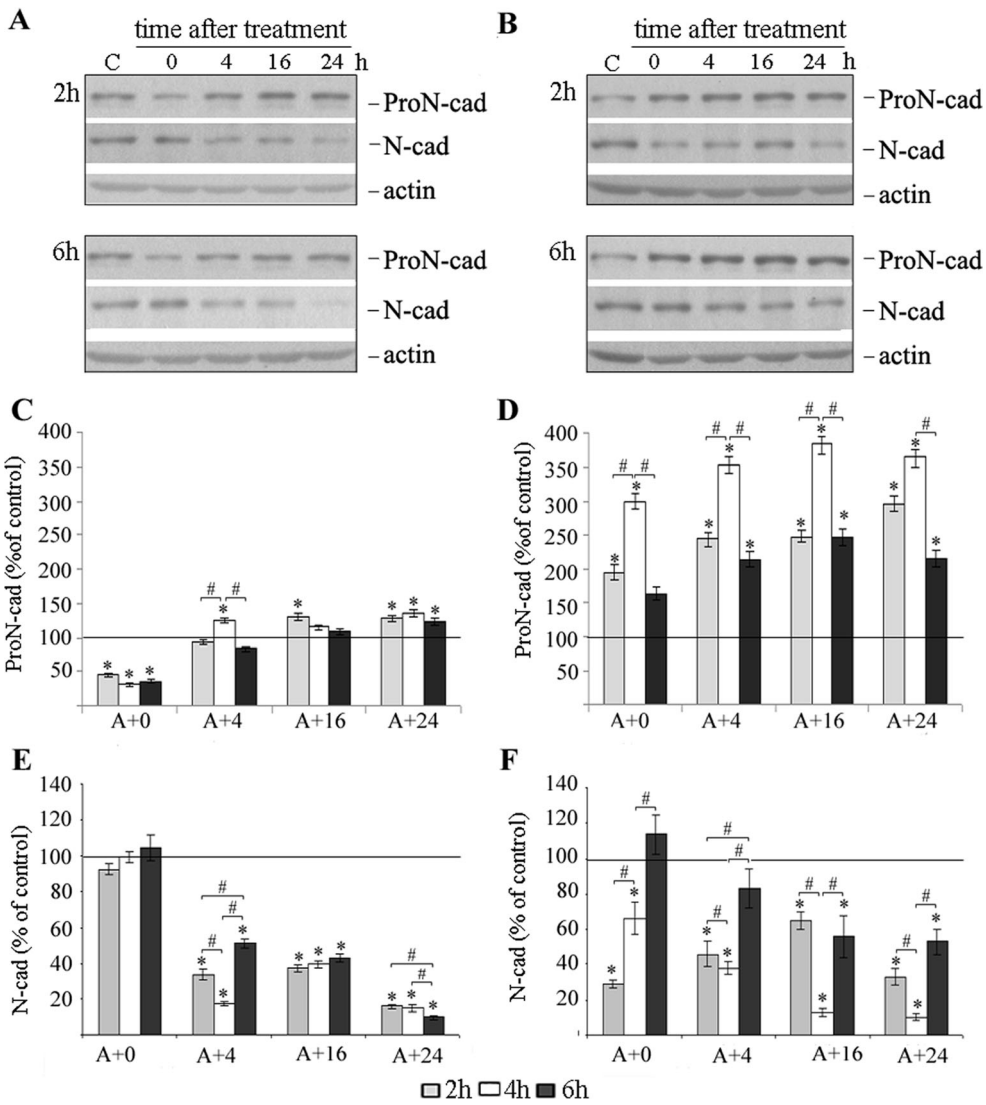

Fig. 5 **a**–**f** Time course of N-cad protein expression in the developing brain after exposure to propofol anesthesia. The levels of N-cad expression were assessed by Western blot analysis of total protein extracts obtained from the cortex (**a**) and the thalamus (**b**) of P7 rats. The results are presented for animals at different recovery time points (0, 4, 16, and 24 h) after exposures to propofol anesthesia for 2, 4, and 6 h. Representative Western blots are shown with protein bands corresponding to ProN-cad, mature N-cad, and β-actin, which served as an internal control of the protein load. *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. other propofol exposures at the same time point

followed by recovery and rise by ~30% above the control value at 16–24 h ($p < 0.05$) post anesthesia. Similar pattern of changes were observed in the thalamus following 2 and 4 h propofol anesthesia (Fig. 5b). However, after a 4-h-long exposure to propofol, a decrease of drebrin at 0 h time point was omitted, while an increase at later time points was more robust compared to other two treatments (50–83% compared to control, $p < 0.05$).

Propofol Treatment Increases MAP-2 Isoforms Expression in the Cortex and Thalamus of P7 Rats

MAP-2 is one of the most important cytoskeleton proteins in dendrites. It is presented in high-molecular weight MAP-2 a/b isoform (270–280 kDa) in the adult brain and in low-molecular weight MAP-2 c/d isoform (~70 kDa) that is characteristic for juvenile brain (Leclerc et al. 1996).

Western blot analysis showed propofol anesthesia affected expressions of both MAP-2 isoforms in the cortex and in the thalamus (Fig. 7). In the cortex, a significant increase of MAP-2a/b isoform (3- to 4-fold elevations above control value) was detected at later time points following a 2-, 4-, and 6-h-long propofol anesthesia (Fig. 7A). In the thalamus, on the contrary, a rise in MAP-2a/b isoform expression was immediate, persistent, and more pronounced compared to the cortex (Fig. 7b). Moreover, the level of this isoform was hardly detectable in the samples of control animals. After propofol anesthesia, a 12- to 18-fold increase was measured at 16–24 h time period post treatments. In parallel to an increase of full-length MAP-2 a/b throughout the 24 h time period, ~70 kDa MAP-2a/b protein fragment, that is product of

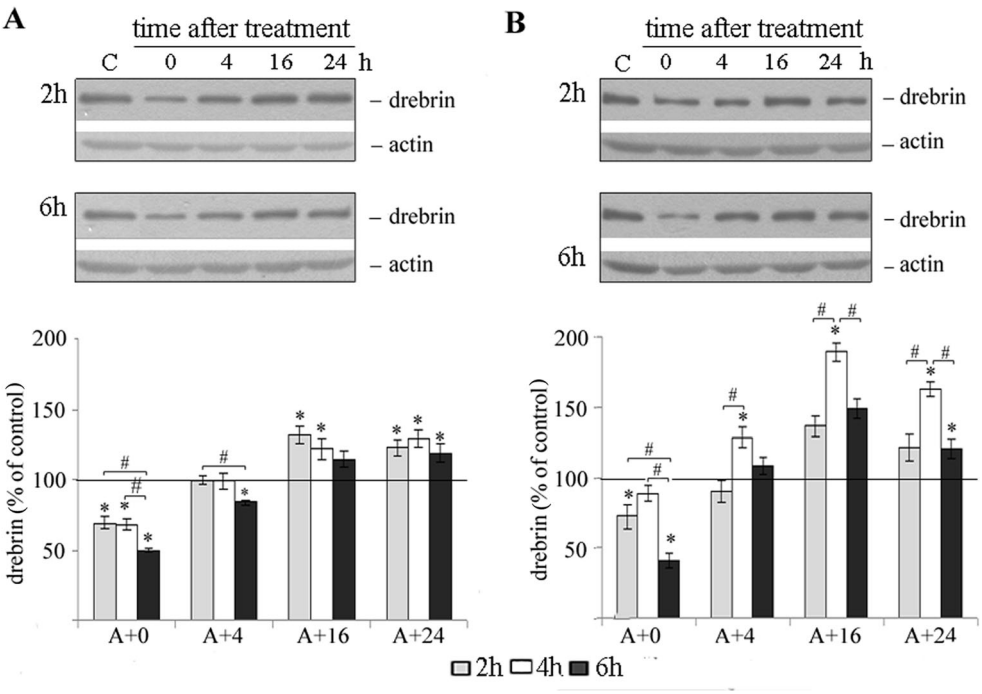

Fig. 6 Time course of drebrin protein expression in the developing brain after exposure to propofol anesthesia. The levels of drebrin expression were assessed by Western blot analysis of total protein extracts obtained from the cortex (**a**) and the thalamus (**b**) of P7 rats. The results are presented for animals at different recovery time points (0, 4, 16, and 24 h) after exposures to propofol anesthesia for 2, 4, and 6 h. Representative Western blots are shown with protein bands corresponding to drebrin and β-actin, which served as an internal control of the protein load. *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. other propofol exposures at the same time point

protease cleavage, declines in both structures (Fig. 6a, b, lower parts of Western blot images).

To examine the expression of MAP-2c/d isoforms that are characteristic and more abundant in the immature brain, we used an antibody that recognizes both adult (mature) and juvenile MAP-2 isoforms. Propofol anesthesia changed MAP-2c/d protein levels in both structures examined (Fig. 7c, d). In the cortex, a gradual increase was measured during the entire period examined and the maximum level (2.4–2.8-fold above control) was measured at 24 h (Fig. 7c). In the thalamus, there was decrease in MAP-2c/d levels immediately following a 2- and 6-h-long anesthesia. However, a decline was followed by recovery and rise of MAP2c/d up to 1.5–1.9-fold above the control level at 24 h time point (Fig. 7D).

Neonatal Exposure to Propofol Anesthesia Changed Motor Activity of 6-Month-Old Rats in the Open Field

Animals that were exposed to a single 6-h-long propofol anesthesia at postnatal day 7 were behaviorally tested at the age of 6 months.

To characterize the locomotor, vertical, and non-locomotor (stereotypic/repetitive) activities, we monitored habituation to the open field during consecutive 3 days of testing (Fig. 8). Regarding locomotor activity, statistical analysis revealed non-significant influence of propofol pretreatment ($F(1, 10) = 2.489$, $p = 0.146$), significant influence of time ($F(2, 20) = 7.398$, $p = 0.004$), and non-significant influence of time × treatment interaction ($F(2, 20) = 0.171$, $p = 0.844$). Post hoc analysis revealed a significant decrease in locomotor activity during the third day of the registration period compared with the first day within both experimental groups (Fig. 7a, $^{\#}p < 0.05$). There were no significant differences between the control and propofol-pretreated groups across days of testing.

Concerning vertical activity of the animals, statistical analysis revealed significant influence of propofol pretreatment ($F(1, 10) = 8.204$, $p = 0.017$) and time ($F(2, 20) = 25.867$, $p = 0.001$) while pretreatment × time interaction was not significant ($F(2, 20) = 0.803$, $p = 0.462$). Post hoc analysis revealed a significant decrease in vertical activity during the second and third day of the registration period compared with the first day within both experimental groups (Fig. 8b, $^{\#}p < 0.05$). There were no statistically significant differences between the control and propofol-pretreated groups across days of testing.

Regarding non-locomotor (stereotypic/repetitive movements) activity, statistical analysis revealed significant influence of propofol pretreatment ($F(1, 10) = 15.561$, $p = 0.003$). Importantly, propofol-pretreated animals showed more intense non-locomotor activity than the control group regarding all 3 days of testing (Fig. 8c, $^{*}p < 0.05$). The influence of time was significant ($F(2, 20) = 4.997$, $p = 0.017$) while pretreatment × time interaction was not significant ($F(2, 20) = 0.002$, $p = 0.998$) when non-locomotor activity was analyzed, as in the case of locomotor activity. Post hoc analysis also revealed a significant decrease in non-locomotor activity during the

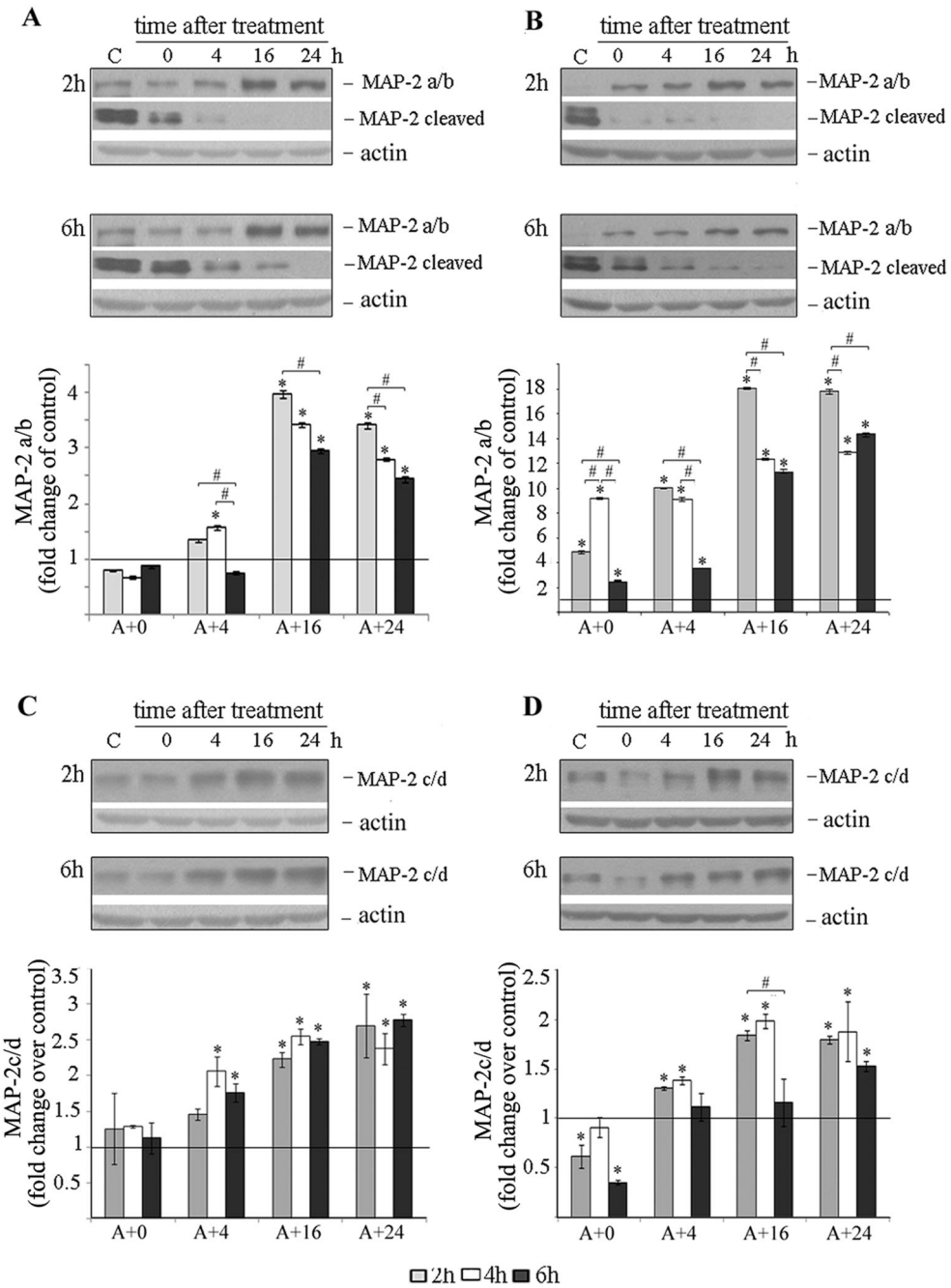

Fig. 7 Time course of MAP-2 isoforms expression in the developing brain after exposure to propofol anesthesia. The levels ofMAP-2 expression were assessed by Western blot analysis of total protein extracts obtained from the cortex (**a**, **c**) and the thalamus (**b**, **d**) of P7 rats. The results are presented for animals at different recovery time points (0, 4, 16, and 24 h) after exposures to propofol anesthesia for 2, 4, and 6 h. Representative Western blots are shown with protein bands corresponding to MAP2a/b (**a**, **b**), MAP2c/d (**c**, **d**), and β-actin, which served as an internal control of the protein load. *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. other propofol exposures at the same time point

third day of the registration period compared with the first day within both experimental groups (Fig. 8c, $^{\#}p < 0.05$).

Neonatal Exposure to Propofol Anesthesia Induced Anxyolitic Effect in 6-Month-Old Rats in the Light/Dark Box

The light/dark test is based on the innate aversion of rodents to brightly illuminated chamber of the apparatus. Statistical analysis revealed significant influence of propofol pretreatment as a factor regarding the duration of time spent in the light chamber and the duration of time spent in the dark chamber (Fig. 9a, b, $^{*}p < 0.05$). The duration of time spent in the door area and the frequency of stretches from the dark chamber into the light chamber were not significantly changed between treated and control group (Fig. 9c, d, respectively).

Discussion

Based upon the known deleterious effects of general anesthetics on neuronal survival and morphology which are well

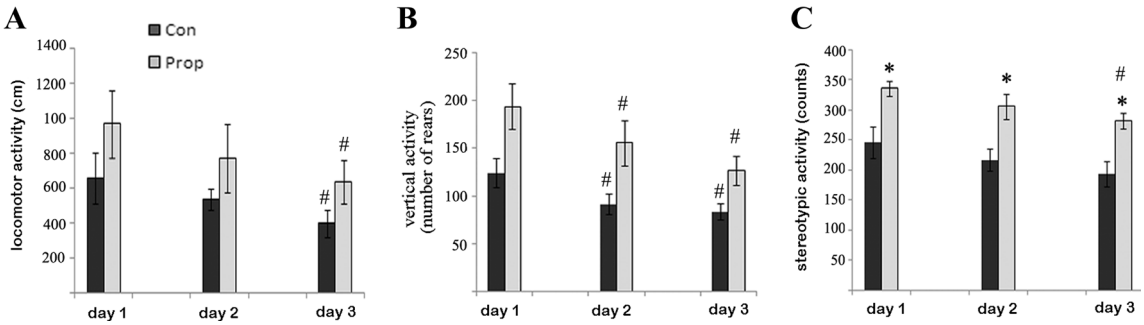

Fig. 8 Motor activity of rats neonatally exposed to propofol anesthesia during habituation testing at P180. Results of locomotor (**a**), vertical (**b**), and stereotypic (**c**) activities expressed as the summary for the whole 30-min registration period to assess inter-session habituation. *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. the same day of control group; $^{\#}p < 0.05$ vs. the first day of the same group

documented mainly by the cyto-histopathological studies (Vutskits et al. 2005; Turina et al. 2008; Jevtovic-Todorovic et al. 2003, Huang and Yang 2015; Lunardi et al. 2015; Sanchez et al. 2011), we hypothesized that propofol could change expression of the key synaptic proteins involved in synaptogenesis, plasticity, and brain development. Therefore, in this study, we analyzed the spatiotemporal expression patterns of presynaptic (GAP-43, synaptophysin, α-synuclein), trans-synaptic (*N*-cadherin), and postsynaptic (MAP-2 and drebrin) proteins in the cortex and thalamus of 7-day-old rats after a single exposure to propofol anesthesia of different duration. The major findings of this study are (1) remarkable, brain region-specific alterations in all examined synaptic proteins that may be molecular substrates underlying structural and functional changes in the synapses and (2) impaired behavioral responses which last even 6 months after a single propofol exposure, suggesting that neonatal propofol anesthesia induced synaptic pathology that, together with other mechanisms previously shown in our work (Milanovic et al. 2010, 2014, 2016), might lead to permanent behavioral consequences extending to mature adulthood and probably beyond.

Presynaptic proteins GAP-43, SPH, and α-SYN have both structural and functional roles in developing axons. GAP-43 is not only associated with neurite outgrowth and formation of novel neuronal connections in developing brain but is also important for nerve regeneration and synaptic plasticity mechanism in the adult central nervous system (Denny 2006). In this study, we have shown that in vivo propofol induced sustained expression of full-length GAP-43. Earlier studies revealed that propofol induces growth cone collapse and neurite retraction in vitro (Turina et al. 2008; Al-Jahdari et al. 2006), suggesting that the elevation we found in the brain could be compensatory, representing a need of developing neurons to maintain/restore existing or to develop new contacts by axonal extensions and synaptic reorganization. The role of GAP-43 is achieved through promotion of local actin filament stabilization that is associated with the formation of filopodia, which is the first step in process of neuronal branching and axonal path finding during development. The expression and function of GAP-43 is highly dependent on protein kinase C-mediated phosphorylation that makes it resistant to calpain proteolysis (Denny 2006). In line with our previous work that showed enhanced calpain activity after propofol anesthesia (Milanovic et al. 2010), we revealed herein enhanced GAP-43 proteolysis during the first hours after anesthesia. The precise role of the proteolytic fragment is not clear, though it was proposed that it prevents complete cleavage of intact GAP-43 by m-calpain as a negative feedback and

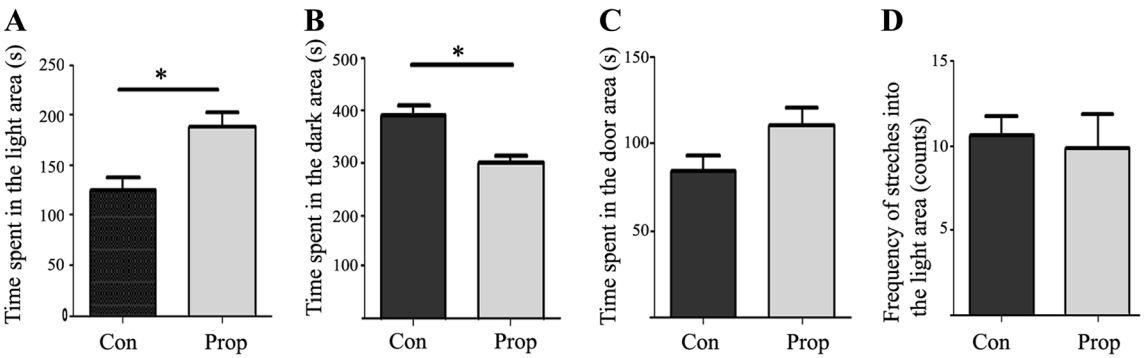

Fig. 9 Effects of neonatal exposure to propofol anesthesia on anxiety-related behaviors of rats in the light/dark box at P180. Results we obtained by measuring the time spent in light (**a**) or dark (**b**) compartment, in the door area (**c**), and by numbers of stretches to the light compartment (**d**). *Bars* indicate the mean ± SEM. $^{*}p < 0.05$ vs. control group

in such a way participates in modulation of neuronal response to repulsive and apoptotic signals (Zakharov and Mosevitsky 2007).

Other two studied presynaptic proteins, synaptophysin and α-synuclein, show similar pattern of changes after propofol anesthesia that is, to a certain extent, expected regarding their colocalization at presynaptic terminals (Murphy et al. 2000), in the membranes (SPH) or in close association with membranes (α-SYN) of neurotransmitter-containing vesicles, implying their role in synaptic vesicles recycling, neurotransmitter synthesis, release, and neurogenesis (Cheng et al. 2011; Bellani et al. 2010). In this study, we found that propofol induced a significant and lasting decrease of SPH and α-SYN in the cortex. As these proteins are located in axonal terminals, their decrease in the cortex may be caused by loss of thalamic neurons whose axons provide the main input to cortical neurons; this is in line with neurodegeneration found in the thalamus in our previous work (Milanovic et al. 2010, 2014) and in other models (Balakrishnan et al. 2013). An observation that propofol strongly modulates synapses in the cortex is also supported with concomitant decline in expression of postsynaptic protein drebrin, as well as of N-Cad, protein that maintains pre- and postsynaptic apposition. SPH has served as a measure of synaptic density (Evans and Cousin 2005; Wallas et al. 1988), and its reduction is in line with studies showing detrimental effects of anesthesia on developing synapse (Zhong et al. 2016; Sanchez et al. 2011; Lunardi et al. 2010). However, upregulation of the presynaptic proteins expressions in the cortex 24 h post anesthesia, as well as in all time points in the thalamus, may be interpreted as a part of plasticity and/or reorganizational response to propofol anesthesia-induced injury, possibly related to modification of vesicular function or trafficking, that determines synaptic efficiency and synaptic strength, respectively (Bellani et al. 2010; Thompson et al. 2006; Evans and Cousin 2005).

N-cadherin is a trans-synaptic cell adhesion protein that bridges the synaptic cleft between two neurons, thus participating in the synapse assembly and the formation of synaptic circuits and synaptic plasticity (Tai et al. 2008). Presynapticaly, N-cad regulates synaptic vesicle recruitment and recycling (Yam et al. 2013) and binds on the outer surface of a growth cone that results in the phosphorylation of GAP-43 and the subsequent changes in the membrane skeleton, causing the growth cone to advance (Dunican and Doherty 2000). Postsynapticaly, N-cad functions in spine remodeling, stabilization, and maturity (Yam et al. 2013; Mendez et al. 2010). N-cad is synthesized as a precursor protein (ProN) that is adhesively inactive until its prodomain is removed by different convertases generating N-cad (Reines et al. 2012). Here, we showed that propofol anesthesia lead to an inverse relationship between expressions of ProN and N-cad proteins. A decline of N-cad can be interpreted as a loss of adhesive interactions between adjacent neurons that leads to destabilization and eventual loss of synapses. ProN inhibits synapse formation (Reines et al. 2012; Latefi et al. 2009; Takeichi and Abe 2005) and its rise following propofol anesthesia enables neurite membrane to pass each other during the process of active axons and dendrite outgrowth and branching, as strong adhesive bonding would not be desirable at that moment. In line with this, our findings may reflect, at the same time, difficulties of immature brain to stabilize synapses, mirrored as decline in N-cad expression, and the advancement of compensatory mechanisms, through ProN rise, to reconnect and maintain synapse number and activity level after propofol anesthesia. This hypothesis is supported by the simultaneous upregulated levels of drebrin and MAP-2 (as discussed below), postsynaptic proteins whose expression mirror the well-being of spines and dendrites, respectively. To the best of our knowledge, results presented here are the first that describe alterations in expression of N-cad in developing brain after application of clinically used anesthetics. Notably, N-cad proteolytic product, that was not examined here, can regulate CREB binding protein (CBP) level (Marambaud et al. 2003) that was recently described as a factor in epigenetic changes induced by neonatal anesthetic (Dalla Massara et al. 2016). This regulation may be of concern in neurodevelopmental disorders like Tourette's syndrome (Sun et al. 2016) and in neurodegenerative disorders like AD (Marambaud et al. 2003) that impose necessity of further studies aimed to define precisely the role of N-cad in anesthesia-induced developmental toxicity.

The impact of propofol anesthesia on postsynaptic neurons was examined through expression of two crucial proteins, drebrin which regulates shape and size of dendritic spines and MAP-2 which plays a key role in morphology and function of dendrites (Koleske 2013; Miller and Kaplan 2003). Drebrin is critical for dendritic spine morphogenesis, as it clusters in filopodia that are precursors of spines, and morphology, as it regulates shape and size of spines determining their stability and maturity (Ivanov et al. 2009; Koleske 2013; Tada and Sheng 2008). The amount of drebrin available is of importance for the homeostatic mechanism that maintains the structural and functional balance between excitatory and inhibitory synapses. Our data showed that propofol produced a dramatic loss (30–50%) of drebrin immediately following termination of anesthesia. This finding supports, on the molecular level, morphometric data from previous studies showing a significant decrease in dendritic spine number and densities even after a single propofol injection (Yu et al. 2013; Briner et al. 2011) or following other anesthetics (Platholi et al. 2014). Furthermore, it has been shown that an antisense-mediated reduction by 40% in drebrin expression is sufficient to induce synaptic dysfunction and strongly correlates with the severity of cognitive impairment accompanying normal aging and neurological disorders, including Alzheimer disease (Counts et al. 2006; Kojima and Shirao 2007; Koleske 2013). On the other hand, not only a spine loss but also an increased stability or density of spines

may be consequences of pathological mechanisms as some mental retardation and neuropsychiatric disease are accompanied with elevated spine numbers (Penzes et al. 2011). In light of that, it is a question whether an increase of drebrin expression we got in our study 16-24 h after anesthesia termination is a plasticity that leads to recovery or maladaptation.

MAP-2, cytoskeleton proteins expressed in dendrites, undergoes developmentally regulated alternative splicing, resulting in a mature isoform, termed MAP-2a/b, that increases during brain maturation, and a juvenile isoform, MAP-2c/d, that declines after postnatal day 10 (Leclerc et al. 1996). The strictly developmentally regulated MAP-2 isoform expression suggests different functional roles for these proteins that were confirmed in non-neural Sf9 cells, where over-expression of MAP-2c/d juvenile form induces multiple short, thin processes that differentiate to dendrites, while adult MAP-2a/b over-expression induced a single process that becomes an axon (Leclerc et al. 1996). We found that propofol anesthesia vigorously increases expression of both mature and juvenile MAP-2 isoforms, especially in the thalamus. Elevation of mature MAP-2a/b may be a consequence of reduced degradation that resulted in higher availability of the intact protein. The role of MAP-2 is to promote assembly and stabilization of microtubules and their interactions with other cytoskeletal proteins or the plasma membrane that may provide the mechanism to withstand initial damage (Fifre et al. 2006). As propofol anesthesia was shown to induce neurodegeneration in the neonatal brain (Milanovic et al. 2010), we suggest that intensive MAP-2 immune-staining observed in this study may be, in part, interpreted as a direct adjustment of the surviving neurons to injury induced by propofol and, to some extent, as a response of neurons to a loss of innervations. The function of juvenile MAP-2c/d isoforms is both to stabilize microtubules and alter actin organization required for neurite initiation (Dehmelt et al. 2003). Therefore, we propose that upregulation of MAP-2c/d following propofol anesthesia might contribute to maintaining primary neurites; this is compatible with previous in vitro and in vivo studies which reported that propofol did not affect the number of primary dendrites in vitro and in vivo (Vutskits et al. 2005; Briner et al. 2011). However, propofol-induced alteration in MAP-2 expression might have detrimental consequences as it could disturb isoform balance in developing brain and switching from juvenile to adult pattern of expression that is most prominent during the second postnatal week (Leclerc et al. 1996). In line with this, significant upregulation in the MAP-2 a/b isoforms is detected in 9-day-old rats in status epilepticus (Jalavaa et al. 2007) and is part of synapto-dendritic abnormalities seen in neuropsychiatric disorders (Cotter et al. 2000).

In our previous work, we have shown that early and prolonged exposure to propofol anesthesia during rat neonatal period resulted in hyperactivity during early adolescence (P30) which persisted, in a more delicate form, till young adulthood (P60) (Milanovic et al. 2016). Though the age range of 8–12 weeks is commonly used in studies is designated as adulthood, mainly based on the sexual maturity of the rodents, many developmental processes are still ongoing at this age (Jackson et al. 2016) and 60-day-old males may be in the gray zone between adolescence and adulthood (Spear 2000; Sengupta 2011). To assess behavioral consequences of neonatal propofol anesthesia at more delayed time points, we performed behavioral testing 6 months (P180) after propofol administration, the age of Wistar rats that is considered as the mature adult. To the best of our knowledge, the effect of propofol on behavior at such delayed time point after single neonatal exposure has not been investigated yet. We found that 6-month-old animals neonatally exposed to propofol had control-like locomotor and vertical activities, but increased number of repetitive/stereotypic-like movements during 3 consecutive days of testing. Although our previous finding showed that hyperactivity could be an obstacle in reaching control-like memory score in adolescence (Milanovic et al. 2016), in the current study, we did not detect difficulties in habituation behavior in mature adult animals but, importantly, decreased anxiety in light/dark box test. Obtained results pointed that behavioral dysfunctions observed in adolescent animals neonatally exposed to propofol anesthesia differentially evolve during adulthood with motor problems becoming dominant over cognitive. Moreover, these findings prompted us to connect hyperactivity during juvenile period with increased stereotypy during late adulthood. Although, from the developmental point of view, motor stereotypes occur commonly in autistic spectrum disorders (ASD) representing external reflection of improper internal neurological maturation (Barry et al. 2011), some recent findings accentuate co-occurrence of attention deficit hyperactivity disorder and ASD in children (Leitner 2014). However, there are no data that address how the relationship between the two disorders changes during the life span. Our previous findings already accentuated neurodegeneration, neuroinflammation, and synaptic remodeling in the cortex and thalamus of neonatal rats exposed to propofol anesthesia, but the degree of contribution of each of mentioned processes within these (and other) brain regions to observed behavioral responses in mature adult animals could not be specified. As stereotypic behaviors are related to situation of altered arousal (Singer 2009), which is highly dependent on the midline thalamus functioning, our findings could reflect a strong impact of neonatal propofol anesthesia on developmental plasticity of the thalamus and consequent inadequate perception of the surroundings, appearance of repetitive movements (with the purpose to compensate for a deficit of external arousal), and decreased anxiety. Indeed, there are indications that some types of repetitive behavior in ASD are not obviously related to anxiety and even seem to be associated with sensory seeking or positive mood states (Boyd et al. 2010). Selection of time end-points for

assessment of functionality as well as different experimental paradigms makes comparison of behavioral results of different studies difficult, but our results are in agreement and qualitatively contributes to the findings in other studies demonstrating long-term behavioral deficits in rodents after neonatal propofol exposure (Karen et al. 2013; Han et al. 2015; Wang et al. 2015; Gonzales et al. 2015; Yu et al. 2013; Gao et al. 2014; Xu et al. 2016). It should be noted that this study, as well as other investigations, does not provide sufficient evidence for a causal relationship between anesthesia-induced changes in synaptic plasticity or neurodegeneration and subsequent behavioral outcome. As the mechanism of anesthesia-induced long-term neurocognitive dysfunction remains elusive, eventual prevention is difficult. However, Shih et al. (2012) suggest post exposure treatment by environmental enrichment as a powerful intervention to improve isoflurane-mediated behavioral dysfunction. These findings may have relevance to neonatal and pediatric anesthesia as it demonstrated that treatment can be effective even when delayed.

Interestingly, all of synaptic plasticity proteins examined here are substrates of calpain and/or caspase-3 (Chimura et al. 2015; Han et al. 2013; Jang et al. 2009; Zakharov and Mosevitsky 2007; Dufty et al. 2007; Hoskison and Shuttleworth 2006), proteases that are activated by propofol, as documented in our previous work (Milanovic et al. 2010). Their proteolysis could initiate local apoptotic signaling at the synapse-synaptic apoptosis that could be transferred to the cell body (Mattson et al. 1998). On the other hand, proteolytic processing could be considered as a controlled structural rearrangement that, through turnover and availability of these proteins, influence or change the functions of synapses. Synthesis, degradation, and trafficking of synaptic proteins are essential for synaptic proteins homeostasis, i.e., proteostasis. At present, it seems that there is still much uncertainty concerning the metabolic turnover of synaptic proteins and much to learn about how these might be affected by physiological and pathological conditions (Rosenberg et al. 2014).

Developing brain is characterized by highly dynamic structural changes of dendrites and potential presynaptic partners that demand large amounts of proteins and lipids, which must be continuously replenished (Sernagor et al. 2010; Koleske 2013). Expression of synaptic plasticity proteins may present a marker of synapse formation, maintenance, and function (Priller et al. 2006; Meyer and Smith 2006; Niell et al. 2004). Considering that more than 96% of dendritic spines have excitatory synapses, it is likely that expression of drebrin, which is located on the spines, reflects alterations in dendritic spine density and the amount of excitatory synaptic inputs (Vutskits 2012). N-cadherin is very abundant protein in PSD (Sheng and Kim 2012), and therefore, the level of its expression can also be used as a marker of excitatory synapse integrity. These observations, accompanied by presynaptic proteins alterations, are in line with previous findings that suggest anesthesia-induced interference with excitatory/inhibitory balance (reviewed at Vutskits 2012). To corroborate influence of propofol anesthesia on inhibitory synapses that are formed mainly on dendritic shafts, specific markers, like gephyrin, or electrophysiological recording would be necessary.

There are few limitations of this study that should be mentioned. The main one is that arterial blood gas analysis was not performed to determine if propofol anesthesia would cause physiological side effects like hypoxia and hypercarbia, which may result in neuronal damage and altered expression of synaptic plasticity proteins. However, the most recent studies established that multiple propofol injections to P7 rats, that reaches 90 mg/kg/day for 7 consecutive days (Wang et al. 2016), or a single high dose of propofol, 50 mg/kg/day for three consecutive days (Chen et al. 2016), does not significantly change blood gas values. Mild respiratory acidosis induced by propofol anesthesia was ruled out as a contributing factor to observed apoptosis, synaptic loss, and long-term cognitive deficits (Chen et al. 2016), in agreement with earlier findings that the 4-h-long neonatal exposure to CO_2 caused widespread brain cell death but did not lead to a long-term cognitive deficits (Stratmann et al. 2010). Given both, (i) a lower single doses of propofol used and (ii) the similarities of synaptic plasticity protein expression patterns between 6- and the shortest, 2-h-long propofol anesthesia (which is unlikely to cause hypoxia), straighten our assumption that propofol exposure, and not disturbed physiological parameters, is the cause of changes reported in our study. The second limitation is that we have not examined dynamics of synaptic protein expressions throughout or at the end of 6 months period. We presumed that new homeostasis might get established during this period, making potential changes undetectable on the protein level but possible mirrored through observed behavioral dysfunctions. The third limitation may be that lipid solvent of propofol has not been used to exclude lipid emulsion influence on different cellular functions. In clinical practice, different commercially available lipid emulsions serve as drug carriers for propofol and other hypnotics and some of them can modulate membrane proteins like NMDA receptors (Weigt et al. 2002). However, the in vivo relevance of this finding remains to be assessed. In some previous studies physiological saline served as a vehicle (De Roo et al. 2009; Fredriksson et al. 2007) while in others, a lipid emulsion was used (Li et al. 2016; Turina et al. 2008; Oscarsson et al. 2001; Nakao et al. 2003; Vutskits et al. 2005) and all of them demonstrated that the lipid vehicle apparently does not produce any effect regardless of the registered effects of propofol (caspase-3 activation, neurite retraction, actin polymerization, c-Fos induction, changes in dendritic spine density and morphology, and neurodegeneration).

Overall, anesthesia can be considered as a risk factor early in life that places an additional burden on the plastic capacity of the developing neural system (Schaefers and

Teuchert-Noodt 2013; Vutskits 2012). Anesthesia-mediated aberrant formation of neural circuits may lead to occurrence of neurodevelopmental disease, like ADHD and autism, while inappropriate maintenance of neuronal circuits may lead to the neurodegenerative diseases (Stoeckli 2012), like Alzheimer's, that are more and more accepted to be synaptic disorders having their origin rooted in development (Schaefers and Teuchert-Noodt 2013). Neuroplasticity may be a link between early life events and neurodegeneration, and in the light of that, the role of anesthetics as synaptotoxic agents needs to be further deciphered.

In summary, our data clearly showed that neonatal propofol anesthesia induced impaired behavioral response in adult mature brains, which might be a consequence of synaptic dysfunction due to the strong alterations of pre-, trans-, and postsynaptic proteins expressions early in the development. We have established overall decrease of SPH, a-SYN, N-Cad, and drebrin, proteins that are found closely on both side of synaptic junctions, that finely indicated impaired and unstable function and structure of the excitatory synaptic contacts immediately after anesthesia cessation, in connection with suppressed neuronal activity. On the other hand, GAP-43 and MAP-2, proteins that participate in the process of neuronal growth cones and dendritic branching that is very dynamic at P7, were preserved/upregulated following propofol anesthesia that could be interpreted as a compensatory mechanism in maintaining structural integrity, enlargement, and stabilization of developing axons and dendritic arbors. We concluded that propofol disturbed a fine balance between stabilization and destabilization mechanisms in vulnerable immature brain which responded by neuroplastic changes that are incorporated permanently into existing networks. Moreover, our results allude that the aberrant developmental metaplasticity, which is form of homeostatic plasticity that tunes magnitude and direction of future synaptic plasticity based on previous neuronal or synaptic activity, could be a base for different behavioral pathologies later in life (Jang and Chung 2016; Schaefers and Teuchert-Noodt 2013).

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Compliance with Ethical Standards All experimental procedures were in compliance with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research, University of Belgrade, and in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH).

Conflict of Interest The authors declare that they have no conflict of interest.

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