Propofol Anesthesia Induces Proapoptotic Tumor Necrosis Factor-α and Pro-Nerve Growth Factor Signaling and Prosurvival Akt and XIAP Expression in Neonatal Rat Brain

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Previously we observed that prolonged exposure to propofol anesthesia causes caspase-3- and calpain-mediated neuronal death in the developing brain. The present study examines the effects of propofol anesthesia on the expression of tumor necrosis factor-α (TNFα), pro-nerve growth factor (NGF), and their receptors in the cortex and the thalamus. We also investigated how propofol influences the expression of Akt and X-linked inhibitor of apoptosis (XIAP) expression, proteins that promote prosurvival pathways. Seven-day-old rats (P7) were exposed to propofol anesthesia lasting 2, 4, or 6 hr and killed 0, 4, 16, or 24 hr after anesthesia termination. The relative levels of mRNA and protein expression were estimated by RT-PCR and Western blot analysis, respectively. The treatments caused marked activation of TNFα and its receptor TNFR-1 and pro-NGF and p75NTR receptor expression. In parallel with the induction of these prodeath signals, we established that propofol anesthesia promotes increased expression of the prosurvival molecules pAkt and XIAP during the 24-hr postanesthesia period. These results show that different brain structures respond to propofol anesthesia with a time- and duration of exposure-dependent increase in proapoptotic signaling and with concomitant increases in activities of prosurvival proteins. We hypothesized that the fine balance between these opposing processes sustains homeostasis in the immature rat brain and prevents unnecessary damage after exposure to an injurious stimulus. The existence of this highly regulated process provides a time frame for potential therapeutic intervention directed toward suppressing the deleterious component of propofol anesthesia.   © 2014 Wiley Periodicals, Inc.

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Recent findings have revealed that clinically used anesthetics induce apoptotic cell death and cell loss when applied to the immature, developing brain during the process of intensive synaptogenesis (Dobbing and Sands, 1979; Jevtovic-Todorovic et al., 2003; Wilder et al., 2009; Briner et al., 2011). Anesthesia-induced apoptosis involves both mitochondrial (intrinsic) and death receptor-mediated (extrinsic) apoptotic pathways, through Bcl-2 family and tumor necrosis factor-α (TNFα)-mediated responses, respectively. Both pathways converge on the activation of the main executor caspase-3, which subsequently cleaves multiple downstream cellular targets (Wajant at al., 2003; Yon et al. 2005; Blomgren et al., 2007). However, apoptosis also can be accomplished via a neurotrophin-dependent pathway. The proform of nerve growth factor (NGF), pro-NGF, preferentially associates with the p75NTR receptor, forming a complex capable of activating the apoptotic cascade in various forms of CNS injury and in neurodegenerative diseases such as Alzheimer's dementia (Chao, 2003; Jevtovic-Todorovic et al., 2003; Pedraza et al., 2005; Volosin et al., 2006; Teng et al., 2010).

There are many physiological control points that protect the cell from inappropriate apoptosis induction, regulating proapoptotic and antiapoptotic activities. Protein kinase B (Akt) may block apoptosis by regulating the expression or activity of the prosurvival or prodeath Bcl-2
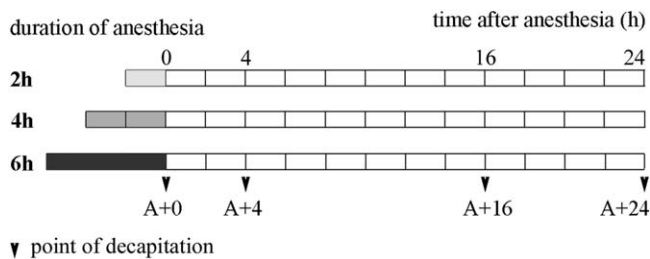
Fig. 1. Timeline of the experiment. Animals were exposed for 2, 4, or 6 hr to propofol by repeated propofol administration (20 mg/kg). Samples were collected at two early (0- and 4-hr) and two late (16- and 24-hr) time points after anesthesia termination.

family members, the functioning of death receptor pathways, and the caspase family proteins (Zhou et al., 2000; Fukunaga and Kavano, 2003; Datta et al., 2008). Akt controls these regulatory points either directly by phosphorylating components of the apoptotic machinery or indirectly by changing the levels of expression of genes that encode for components of the death machinery (Datta et al., 2008).

The other well-known regulators of apoptotic signaling pathways belong to the inhibitors of apoptosis (IAP) family of proteins. Of the eight members of the IAP family, the most potent suppressor of apoptosis is XIAP (Yousefi et al., 2003; Villapol et al., 2008). XIAP inhibits apoptosis by directly binding and inhibiting caspase-3, -7, and -9 (Deveraux et al. 1998). Involvement of XIAP in mechanisms that underlie the neuroprotective effect, leading to reduced apoptosis, has been demonstrated in neonatal brains and in vitro (Korhonen et al., 2001; Wang et al., 2004; Sheth et al., 2009; Li et al., 2010).

In as much as exposure to general anesthesia cannot be avoided when a child's well-being is in danger, a considerable effort has been made in recent years to elucidate the mechanisms of anesthesia-induced developmental apoptosis. At present, there are no data that reliably define the least toxic anesthetic agent, safe and toxic thresholds, appropriate lengths of exposure at specific stages of development, or whether one long exposure is more appropriate than repeated short exposures. Propofol (2,6-diisopropylphenol) is a widely used anesthetic agent applied for the induction and maintenance of general anesthesia in clinical practice (Loepke and Soriano, 2008). Therefore, it is of some interest to assess the effects of the length of exposure to propofol anesthesia on the signaling pathways lying upstream of caspase-3 activation. The present study was undertaken to investigate the time course and the duration of the effects of propofol administration on the proapoptotic molecules pro-NGF and TNFα and their receptors, by examining the relative expression of the mRNAs and proteins in the cortex and the thalamus of P7 rats. We also assessed the induction of pAkt and XIAP, proteins that promote cell survival by inhibiting cell death and consequently prevent neurodegeneration of the immature rat brain. Although the pre-

cise molecular mechanisms of propofol toxicity are unknown, it is apparent that immature neurons upregulate pAkt and XIAP proteins in response to increased pro-NGF and TNFα expression to maintain homeostasis and initiate the survival process.

MATERIALS AND METHODS

Animals and Treatment

Seven-day-old (P7) male Wistar rats (average body weight 12–14 g) were used in all experiments. The procedures were designed to minimize the suffering of the animals and the number of rats used. Rat pups were placed in a temperature-controlled incubator set to an ambient temperature of 35–36°C. Animals not designated to be killed immediately after the anesthesia treatment were allowed to recover in the incubator for 1 hr and were returned to their mothers to feed. Propofol manufactured for intravenous human use was administered to the animals (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.

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 by the University of Virginia Animal Care and Use Committee and were in accordance with the NIH *Guide for the care and use of laboratory animals*.

Experimental Procedures

To prevent possible litter effects, in all circumstances a single pup from a litter was assigned to every single group of experimental rats mentioned at the appropriate points in this article. Propofol was administered intraperitoneally to P7 rat pups (n = 92) in two, four, or six bolus injections of 20 mg/kg at 1-hr intervals to achieve propofol anesthesia, designated as 2-, 4-, or 6-hr anesthesia (Milanović et al., 2010). The animals were decapitated either immediately after cessation of the exposure times (designated as the 0-hr time point) or after the recovery periods that lasted 4, 16, or 24 hr after termination of propofol exposure (and are referred to as 4-, 16-, and 24-hr time points, respectively; Fig. 1). Control, sham-treated animals received intraperitoneal injections of physiological saline at 60-min intervals. The cortex and the thalamus from both hemispheres were isolated and frozen for subsequent use for RNA and protein analysis (n = 4 per group). Whole brains were isolated (n = 3 per group) for histological studies.

RNA Isolation, Reverse Transcription, and Semiquantitative PCR

Total RNA was isolated from the cortex and the thalamus with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription (RT) reactions were performed with 5 μg total RNA by using oligo-dT primers and M-MLV reverse transcriptase according to the manufacturer's instructions. Primer sequences and PCR

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

Gene	Primers (5′–3′)	Size (bp)	PCR profile	No. of cycles
TNF-α	GCCCTAAGGACACCCCTGAGGGAGC TCCAAAGTAGACCTGCCCGGACTCC	158	94°C, 15 sec; 58°C, 30 sec; 72°C, 30 sec	28
TNFR 1	TGGTGCTCCTGGCTCTGCT ACCTGGAACATTTCTTTCCGA	298	94°C, 15 sec; 58°C, 30 sec; 72°C, 30 sec	26
β-actin	TGGACATCCGCAAAGACCTGTAC TCAGGAGGAGCAATGATCTTGA	142	Depends on target gene	
GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	306	Depends on target gene	

conditions are shown in Table I. PCRs were performed with the GeneAmp1 PCR System 9700 (Applied Biosystems, Foster City, CA). All PCRs were performed from two independent RT reactions, with at least two repeats for each reaction. 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 (Gel Doc 1000; Bio-Rad Laboratories, Hercules, CA) was used for densitometry analysis.

RT and Real-Time RT-PCR

RT reactions were performed in 20 μl by using a high-capacity cDNA archive kit according to the manufacturer's instructions. The reactions were carried out under RNase-free conditions at 25°C for 10 min and at 37°C for 2 hr. The cDNA was stored at −20°C until further use. Relative quantification of NGF mRNA was performed by real-time RT-PCR with the TaqMan assay (ID Rn01533872; Applied Biosystems). GAPDH was included as an endogenous control to correct for differences in interassay amplification efficiency (ID Rn99999916_s1; Applied Biosystems). 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 in RQ Study add-on software for the 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System; Applied Biosystems), with a confidence level of 95% ($P < 0.05$).

Tissue Extracts

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 14,000g 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 microprotein assay (Micro BCA protein assay kit; Pierce, Rockford, IL), with albumin as standard.

Western Blot Analysis

Proteins (20–40 μg per lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Bioscience, Otelfingen, Switzer-

land). The membranes were blocked at room temperature for 1 hr in 3% bovine serum albumin in Tris-buffered saline/0.1% Tween 20 (TBS-T), followed by incubation for 2 hr or overnight with primary antibodies diluted in 1% bovine serum albumin/TBS-T. The immunoblots were processed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T for 1 hr at room temperature. 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. Films were densitometrically analyzed in the computerized image analysis program ImageQuant 5.0. The following antibodies were used: rabbit polyclonal NGF that recognized pro-NGF (25 kDa) and mature NGF (13 kDa), anti-p75, anti-TNFα, rabbit polyclonal P-Akt1/2/3, Thr-308-R, XIAP polyclonal antibody (all from Santa Cruz Biotechnology), and rabbit polyclonal β-actin (ICN).

Immunohistochemical Analysis

The animals (n = 3 per group) were killed 24 hr after the propofol anesthesia. The brains were removed and fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were cryoprotected by three 24-hr incubations at 4°C in solutions of increasing sucrose concentration (10%, 20%, and 30%) in PBS. The brains were frozen in isopentane and stored at −80°C. Every fifth coronal section (18 μm thick) was mounted on the slides and allowed to dry overnight and then stored at −20°C. Sections were immunostained with rabbit anti-XIAP antibody (1:50) in 0.01 M PBS at 4°C overnight and washed, followed by incubation with a secondary fluorescein isothiocyanate-conjugated anti-rabbit antiserum (diluted 1:200; Dako, Glostrup, Denmark) for 2 hr at room temperature. Sections were coverslipped with Vectashield mounting medium with DAPI and analyzed by using an Axio Observer Microscope Z1 (Zeiss, Gottingen, Germany). A negative control slide for each tissue was incubated with nonimmunized horse serum to replace the primary antibody.

Statistical Analysis

Semiquantitative evaluation of the protein levels detected by immunoblotting was performed by densitometric scanning in the computerized image analysis program ImageQuant 5.0. The data are presented as percentages (mean ± SEM) relative to the control samples (assumed to be 100%). Differences between the

experimental groups were tested by using Kruskal–Wallis nonparametric ANOVA with Mann-Whitney's post hoc U test (Statistica version 5.0; StatSoft, Tulsa, OK). Significance was accepted at $P < 0.05$.

RESULTS

Propofol Treatment Increased TNFα mRNA and Protein Expression in the Cortex and the Thalamus of P7 Rats

To examine the effect of propofol treatment on the immature rat brain, P7 pups were exposed to propofol for 2, 4, or 6 hr and decapitated at two early (0- and 4-hr) and two later (16- and 24-hr) time points after termination of each exposure. Because TNFα is the main trigger of the extrinsic apoptotic pathway, RT-PCR and Western blotting were performed to examine the contribution of TNFα to propofol-induced toxicity in P7 brains. Quantification of the mRNA levels revealed significant upregulation of TNFα gene expression in both structures (Fig. 2A,B). The most robust changes, observed as three- to sixfold increases, were detected in the cortex within 4 hr after termination of all three exposures to propofol. The effect of propofol anesthesia on TNFα mRNA expression in the thalamus was even stronger (up to a tenfold increase was detected) and persisted throughout the 24-hr time frame (Fig. 2B).

The TNFα protein level was significantly increased in the cortex immediately after cessation of all three propofol exposures (Fig. 2C). At 24 hr postanesthesia, the level of TNFα protein had declined to about 50% of the control value in all three anesthesia regimens ($P < 0.05$). The changes in the thalamus were more robust, indicated by the higher degree of increase in TNFα protein expression and by the more persistent changes that were registered throughout the examined times (Fig. 2D).

Propofol Treatment Increased TNFR–1 mRNA Expression in the Cortex and the Thalamus of P7 Rats

Semiquantitative RT-PCR analysis revealed that TNFR–1 gene expression increased (up to twofold; $P < 0.05$) in a manner dependent on the duration of propofol anesthesia, with both brain structures displaying similar time profiles (Fig. 3A,B).

Propofol Treatment Changed NGF mRNA and pro-NGF Protein Expression in the Cortex and the Thalamus of P7 Rats

NGF has important roles in both prosurvival and proapoptotic signaling pathways. RT-PCR revealed that the propofol anesthesia affected NGF gene transcription in both structures (Fig. 4A,B). Significant increases were observed in the cortex immediately after cessation of the 6-hr exposure (up to 2.2-fold; $P < 0.05$), and for 45–67% after all three exposures at 24 hr after anesthesia termination. In the thalamus, the strongest upregulation of NGF mRNA (a threefold increase) was induced by the 6-hr propofol anesthesia.

Western blot analyses revealed that the propofol treatment altered the expression of pro-NGF in the cortex and the thalamus of P7 rats (Fig. 4C,D). In the cortex, no changes in pro-NGF levels after the 2- and 4-hr propofol anesthesia were detected. The 6-hr anesthesia induced an increase in the level of pro-NGF (to about 67% compared with the control; $P < 0.05$) only at the 0-hr time point. More than a twofold increase in pro-NGF protein level was observed in the thalamus in an anesthesia duration–dependent manner.

Propofol Treatment Increased p75^NTR Protein Expression in the Cortex and the Thalamus of P7 Rats

The p75^{NTR} receptor protein is a key element in the pro-NGF-mediated apoptotic pathway. Western blot analysis was used to examine its expression in the immature brain after propofol anesthesia (Fig. 5A,B). In the cortex, both the 2- and the 4-hr propofol anesthesia increased p75 levels by 30% at distinct time points. The earliest and longer lasting change was observed after the 6-hr anesthesia (increases of 40–65% compared with the control; $P < 0.05$). In the thalamus, a gradual increase of p75 levels peaked at 24 hr after termination of all three propofol exposures (32–48% compared with the control; $P < 0.05$).

Propofol Treatment Increased pAkt Protein Expression in the Cortex and the Thalamus of P7 Rats

The expression profile of the prosurvival Akt kinase obtained by Western blotting is presented in Figure 6A,B. The highest increase (twofold compared with the control level; $P < 0.05$) was observed immediately at 0 hr and 24 hr after anesthesia. A similar degree of change (approximately twofold) was observed in the thalamus within 0–4 hr after anesthesia (Fig. 6B; $P < 0.05$). More pronounced increases in Akt phosphorylation occurred at later times. Three- to sixfold increases in pAkt expression ($P < 0.05$) were measured after termination of the 2-, 4-, and 6-hr propofol anesthesia.

Propofol Treatment Increased XIAP Protein Expression in the Cortex and the Thalamus of P7 Rats

XIAP is a prosurvival protein capable of blocking activated caspases. Western blot analysis revealed significant changes in XIAP protein expression after propofol anesthesia. In the cortex, the propofol anesthesia induced a slight but significant upregulation (about 30–50%; $P < 0.05$) in XIAP protein levels after the 2- and 6-hr exposures at the later time points (16 and 24 hr; Fig. 7A). In the thalamus, the 2-hr anesthesia induced a significant, 50% decrease in XIAP protein level immediately after termination of anesthesia (Fig. 7B). This was
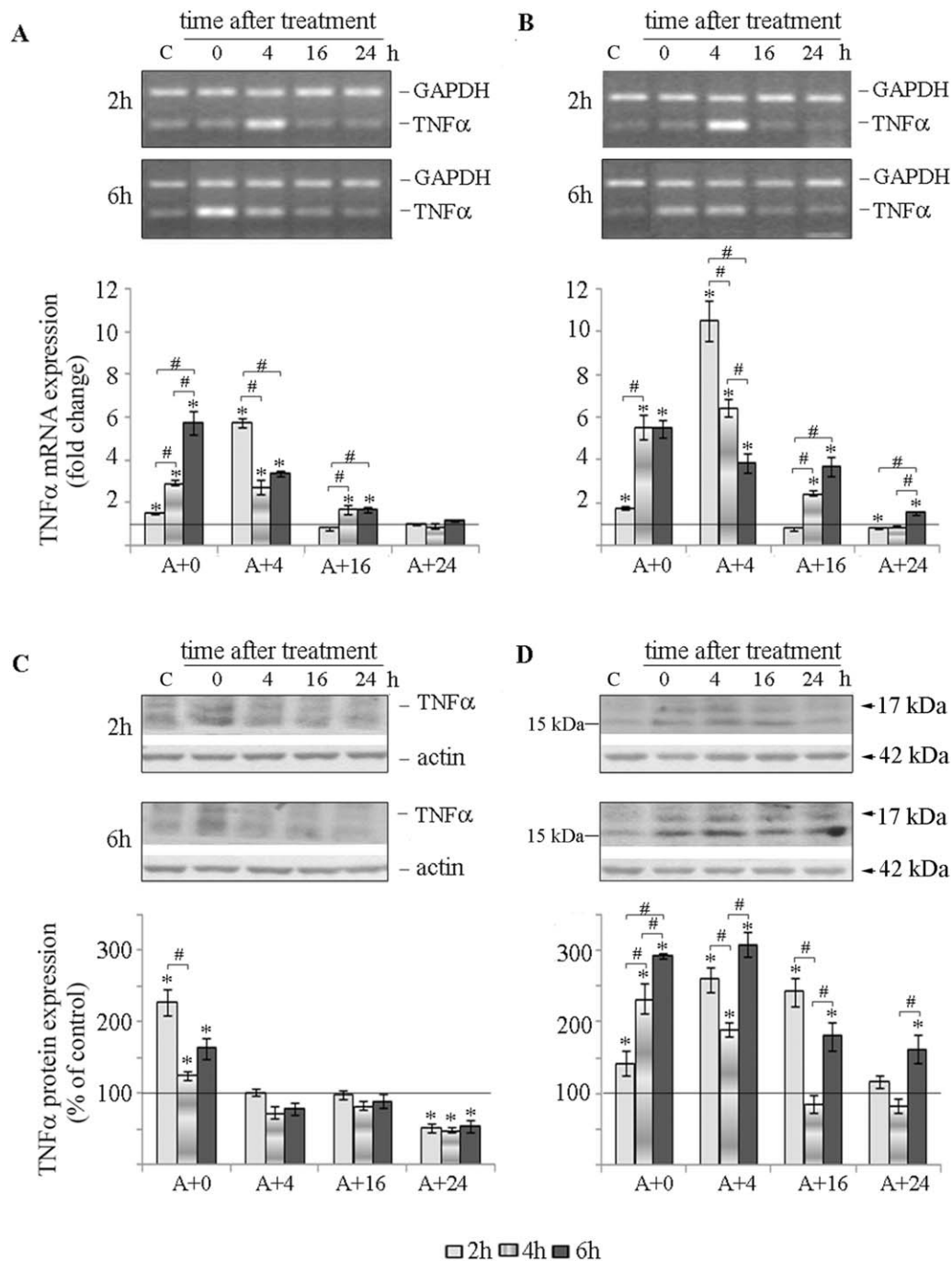
Fig. 2. Time course of TNFα mRNA and protein expression in the cortex (**A,C**) and the thalamus (**B,D**) of P7 rats after 2-, 4-, and 6-hr exposures to propofol. The results are presented for animals at different recovery time points (0, 4, 16, and 24 hr) after exposure to propofol. The panels show representative PCR data for the 4-hr anesthesia. The levels of TNFα protein expression were assessed by Western blot anal- ysis of total protein extracts obtained from the cortex (C) and the thal- amus (D). The results were normalized to the appropriate internal controls, GAPDH for the RNA and β-actin for the protein. Control, (C) sham-treated animals received intraperitoneal injections of physio- logical saline. The results are mean ± SEM. ★$P < 0.05$ vs. the control value, presented as a black line; [#]$P < 0.05$ between treatments.

followed by recovery and an 83% ($P < 0.05$) increase above the control value at 24 hr after anesthesia. The most pronounced change in the thalamus was observed as elevated expression of XIAP after the 4-hr anesthesia,

when it ranged from 75% to 200% ($P < 0.05$) relative to the control level.

To confirm prominent XIAP expression, we also performed immunohistochemical analysis of brain sections
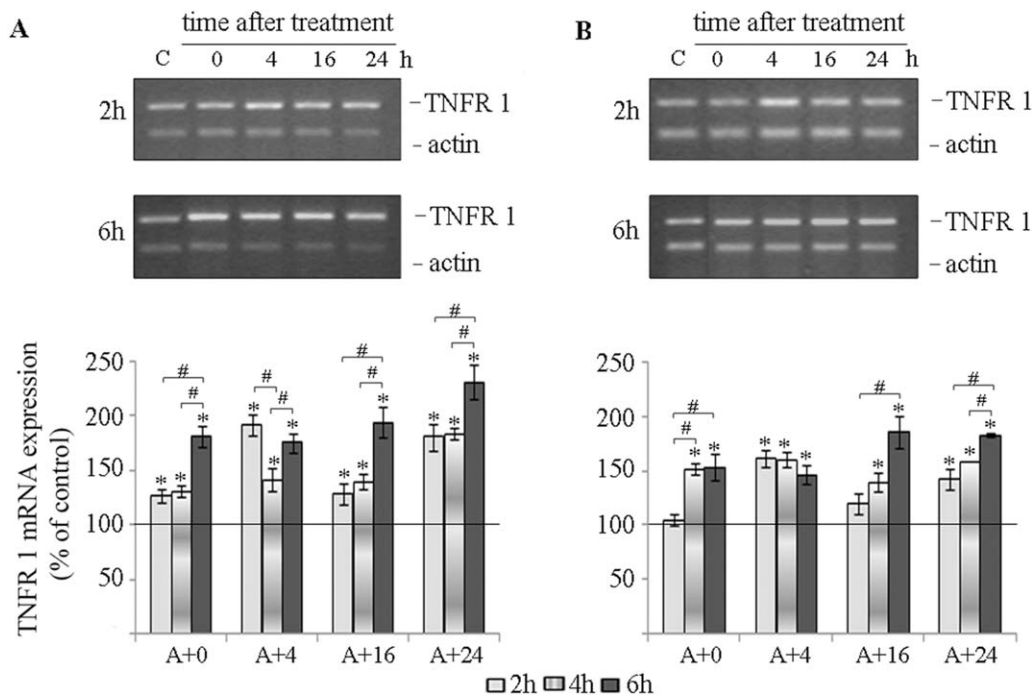
Fig. 3. Time-dependent changes in TNFR-1 mRNA expression in the cortex (**A**) and the thalamus (**B**) of P7 rats after propofol exposure. The results are presented for animals at different recovery time points (0, 4, 16, and 24 hr) after exposure to propofol for 2, 4, and 6 hr. The panels show representative PCR data for the 6 hr anesthesia. The results were normalized to the internal control (β–actin) and are expressed as mean $\pm$ SEM. $\star P < 0.05$ vs. the control value, presented as a black line; $^{\#}P < 0.05$ between treatments.

(Fig. 7C). A marked increase in XIAP was made evident by the appearance of intense XIAP-specific immunoreactivity 24 hr after termination of the 6-hr anesthesia in the thalamus. XIAP immunoreactivity was localized predominantly in the perinuclear region in the thalamus of control animals. At 24 hr postanesthesia, the intensity and pattern of XIAP expression was altered, exhibiting a strong and diffuse pattern in the whole cell.

DISCUSSION

This study demonstrates that prolonged propofol anesthesia induces increased expression of proapoptotic TNFα and pro-NGF mRNAs and proteins in cortical and thalamic structures in P7 rats. We also observed increased expression of pAkt and XIAP proteins that could serve to inhibit cell death and promote survival in the cell's attempt to maintain homeostasis after propofol anesthesia.

We previously described propofol neurotoxicity in P7 rats. We showed that extended propofol exposure, produced by the administration of 2, 4, or 6 propofol injections, induced increased caspase-3 and calpain activities that resulted in neurodegeneration at specific time points (Milanović et al., 2010). The present study revealed a very rapid activation of TNFα at both the mRNA and the protein levels within 4 hr after all three anesthesia regimens. The increase in TNFα expression

coincided with the emergence of apoptotic neurons that were previously detected by Fluoro Jade staining after single and repeated administrations of propofol (Bercker et al., 2009; Pesic et al., 2009; Milanovic et al., 2010). In contrast to the TNF ligands whose expression was reversed to, or below, the control level, transcription of its receptor, TNFR-1, was elevated throughout the observation period after all three exposures to propofol. This result is in agreement with other studies showing differentially regulated expression of cytokines and their receptors (Vitkovic et al., 2000; Sairanen et al., 2001; Perry et al., 2002). We speculate that the increased expression of TNFα and its receptor served as the main mediators of propofol neurotoxicity.

Apoptosis can also proceed via the NGF-p75$^{\text{NTR}}$-mediated cascade. The detrimental role of pro-NGF has been emphasized in studies in which a functional block of the NGF prodomain significantly reduced cell death (Harrington et al., 2004; Volosin et al., 2006). Our results show that propofol anesthesia induced increases in NGF mRNA and pro-NGF protein expression in brain-structure-dependent, and duration of propofol anesthesia-dependent, manners. The changes in NGF and p75$^{\text{NTR}}$ expression were most prominent after the longest, 6-hr, anesthesia. The thalamus appeared to be more responsive to propofol action, but p75$^{\text{NTR}}$ expression changed earlier and was more pronounced in the cortex. A similar
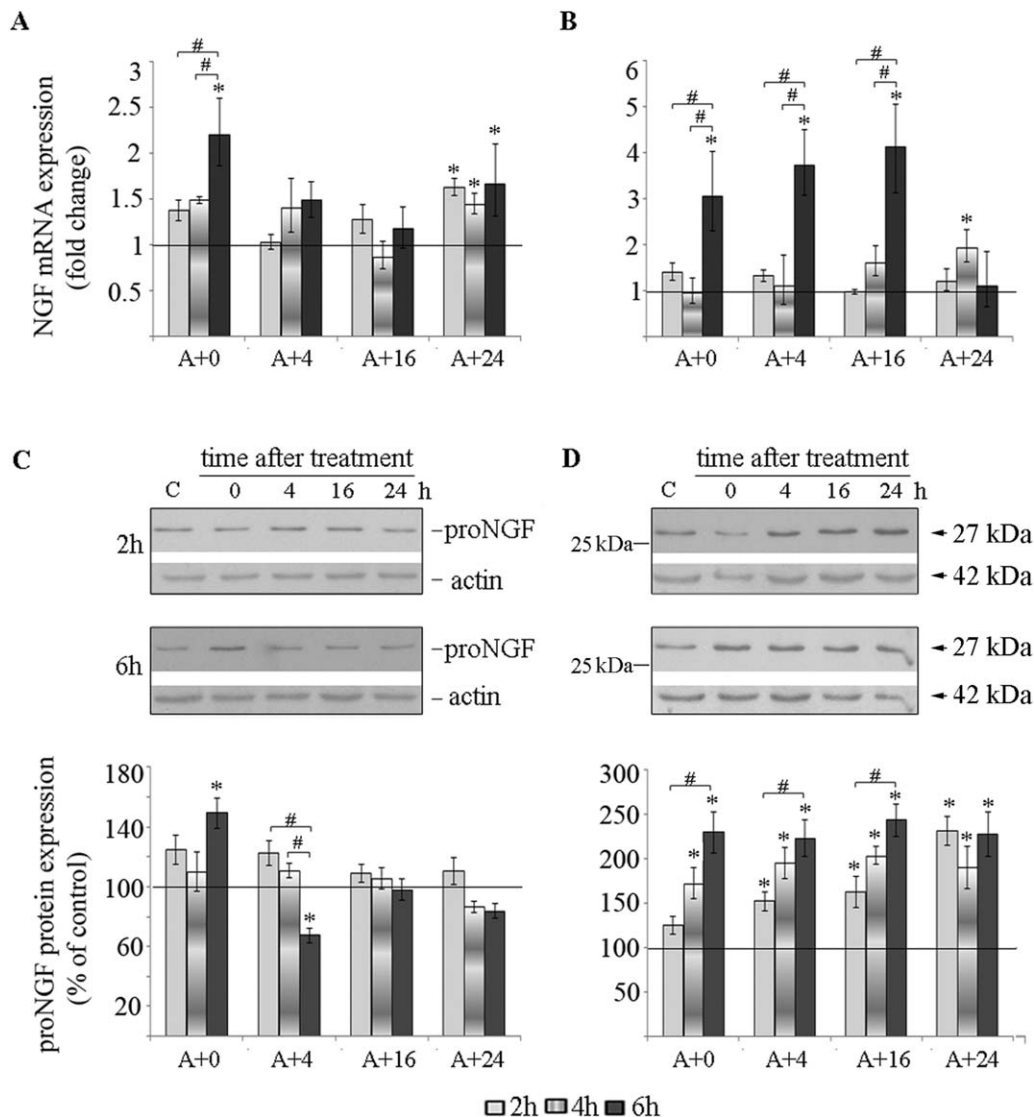
Fig. 4. Time course of NGF mRNA and pro-NGF protein expression. NGF mRNA expression in the cortex (**A**) and the thalamus (**B**) of P7 rats after propofol anesthesia was examined by using quantitative real-time RT-PCR. The results are presented for animals at different recovery time points (0, 4, 16, and 24 hr) after exposure to propofol for 2, 4, and 6 hr. Histograms represent mRNA levels expressed as the fold change relative to the control value obtained from three separate experiments. The level of pro-NGF protein expression was determined by Western blot analysis of total protein extracts obtained from the cortex (**C**) and the thalamus (**D**). A representative Western blot is shown with a single protein band (27 kDa) corresponding to pro-NGF and β-actin, which served as an internal control of the protein load. Histograms represent protein levels expressed as fold changes relative to the control value obtained from five separate experiments. The results are mean ± SEM. *$P < 0.05$ vs. the control value, presented as a black line; #$P < 0.05$ between treatments.

finding (Lu et al., 2006) was considered to be the result of the increased vulnerability of the cerebral cortex at P7 because of different ontogeny, i.e., the different intensities of synaptogenesis in different brain regions that define their critical period of vulnerability to anesthesia (Das et al., 2001). The anesthesia-induced activation of p75[NTR] in the brain has been described recently; however, pro-BDNF, and not pro-NGF, as a ligand was investigated (Head et al., 2010; Pearn et al., 2013). The anesthesia-induced reduction in neuronal activity disturbs the conversion of pro-BDNF to mBDNF, favoring pro-BDNF overexpression and its binding to the p75[NTR] receptor. This sequence of events activates RhoA GTPase, causes a disturbance of the cytoskeleton and leads to subsequent neuroapoptosis (Bjornstrom et al., 2008; Pearn et al., 2012). It was suggested that p75[NTR]-mediated apoptosis is inhibited by the coactivation of Trk receptors (Ibanez and Simi, 2012). However, we found a significant downregulation of p-Trk (up to 70% compared with the control) in the cortex for up to 24 hr after
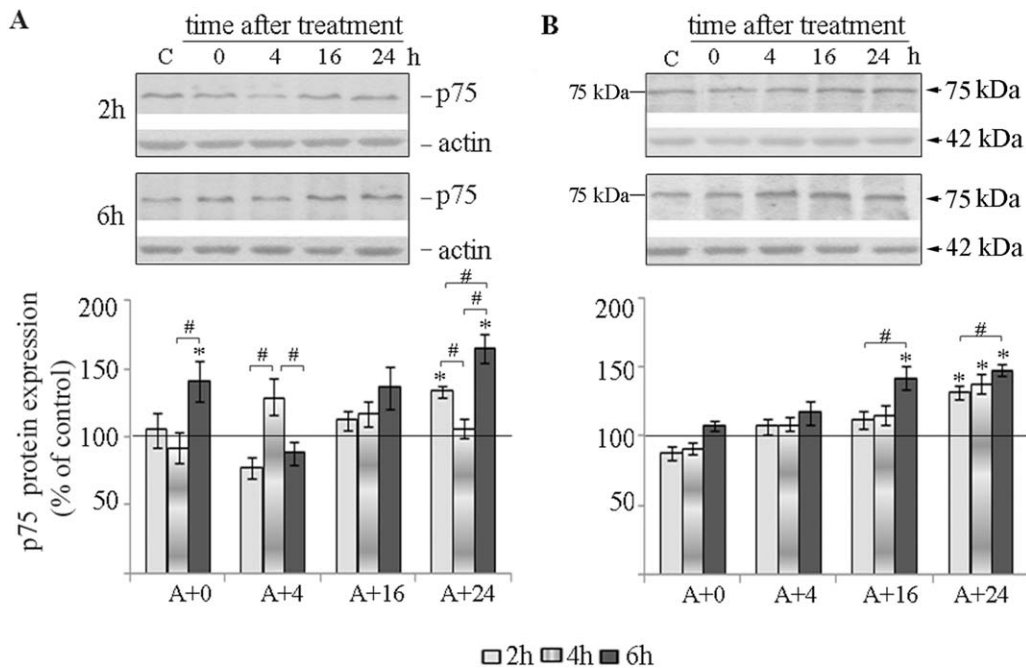
Fig. 5. Time course of changes in p75[NTR] protein levels in the cortex and the thalamus of P7 rats after exposure to propofol. Western blot analysis of total protein extracts obtained from the cortex (**A**) and the thalamus (**B**) was performed. Results are presented for animals at different recovery time points (0, 4, 16, and 24 hr) after exposure to propofol. The panels show representative immunoblots obtained after 2- and 6-hr propofol anesthesia. Results were normalized to the internal control (β-actin) and are expressed as mean ± SEM. $\star P < 0.05$ vs. the control value, presented as a black line; $^{\#}P < 0.05$ between treatments.

the longest exposure (Supp. Info. Fig. 1). Thus, our findings suggest that increased pro-NGF preferentially binds to p75[NTR] and participates in the deleterious effects of propofol anesthesia.

Notably, previous studies suggested that anesthetic neuroapoptosis is limited to rodents younger than 14 days of age and affects predominantly the neocortex and the thalamus. However, the newest data suggest that a period of vulnerability follows the regional peaks in neurogenesis that differ among brain regions. Therefore, detrimental effects of anesthetics can extend at least into young adulthood in brain regions with continued neurogenesis, such as hippocampal dentate gyrus (Hofacer et al., 2013; Krzisch et al., 2013; Deng et al., 2014). Impaired survival and maturation of adult-borne hippocampal neurons, by hindering their subsequent functional integration into the hippocampal circuitry, might play a pivotal role in cognitive dysfunction after propofol anesthesia exposure in adults (Krzisch et al., 2013).

Rescuing cells from propofol-induced brain injury would probably require effective inhibition of death effectors pathways and/or induction of the prosurvival proteins, such as Akt and the IAP family of proteins. Akt protects cells from apoptosis via stimulation of the expression of proteins that favor cell survival (CREB and NF-κB transcriptions factors, Bcl-2 family members, caspase inhibitors c-IAP and XIAP) and by inhibiting proapoptotic proteins (caspase-3, caspase-9, FOXO; Zhou et al.,

2000; Datta et al., 2008). In the present study, we observed an increase in Akt phosphorylation at Thr-308 at later times, i.e., between 16 and 24 hr after the last propofol injection, in both brain structures. These results are not in complete agreement with most other studies, which have described the downregulation of Akt after the application of a mix of anesthetics (midazolam, isoflurane, nitrous oxide) and a single dose of propofol to P7 rats (Lu et al., 2006; Pesic et al., 2009; Karen et al., 2013). The reported decrease in Akt was hypothesized to be caused by an anesthetic-induced depression of neuronal activity that impacted BDNF synthesis, survival, and secretion and impaired the downstream Akt prosurvival signals. We detected a decrease in BDNF mRNA and protein expression (data not shown) and a reduced level of activated neurotrophin receptor p-Trk A (Supp. Info. Fig. 1). Therefore, it is possible that the activation of Akt was mediated not exclusively through BDNF-Trk signaling but through some other mechanism. A moderate increase in cytoplasmic calcium levels could activate Akt by phosphorylation at Thr-308 via the Ca^{2+}/calmodulin complex (Xu et al., 2007). This is in agreement with our previous study in which the increase in the activity of calpain, a Ca^{2+}-dependent protease, was observed to be linked indirectly with a rise in intracellular calcium (Kahraman et al., 2008; Milanović et al., 2010). In addition, several groups have reported an increase in pAkt levels in P7 and P14 animals in a brain-structure-specific manner after
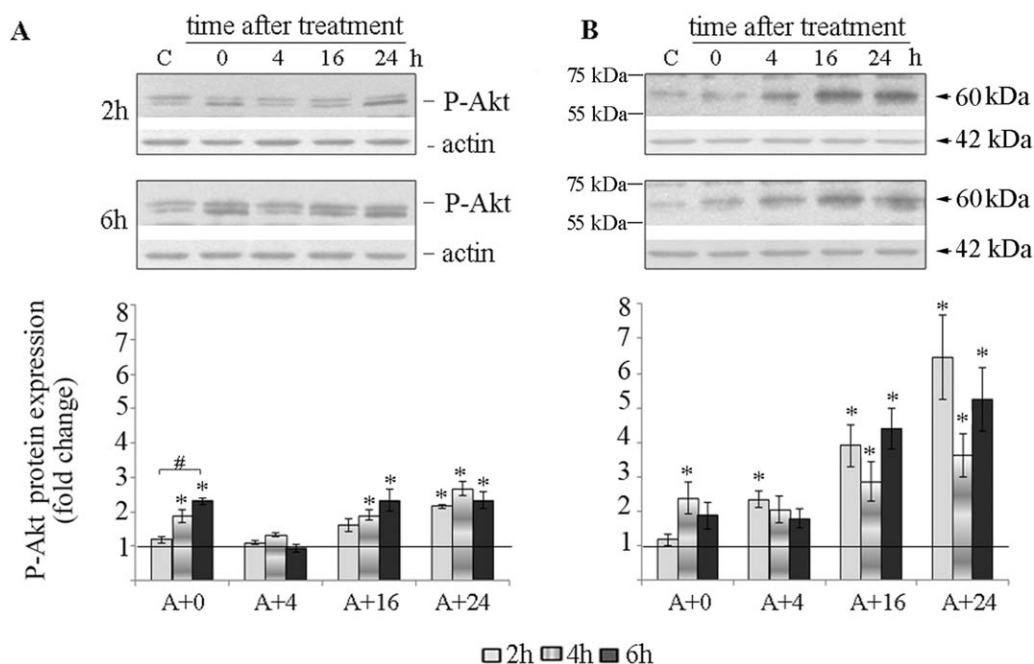
Fig. 6. Time course of appearance of pAkt during propofol exposure in P7 rats. Western blot analysis was used to determine the expression of pAkt (Thr 308) kinase in the cortex (**A**) and the thalamus (**B**). Each graph is accompanied by representative immnoblots obtained after 2- and 6-hr propofol anesthesia. Results were normalized to the internal control (β-actin) and are expressed as mean ± SEM. $\star P < 0.05$ vs. the control value, presented as a black line; $^{\#}P < 0.05$ between treatments.

acute or repeated propofol administration (Popic et al., 2012; Karen et al., 2013).

Whereas Akt functions at the pre- and postmitochondrial levels (Zhou et al., 2000), XIAP plays key roles in cell survival by modulating death-signaling pathways at the postmitochondrial level (Korhonen et al., 2001; Wang et al., 2004). Our findings show for the first time the increase in XIAP protein expression in the developing brain after anesthesia. XIAP has been observed to bind and directly to inhibit caspase-3, −7, and −8 (Deveraux et al., 1998). The neuroprotective effect of XIAP also may involve reduced calpain activity and stabilization of the calpain/calpastatin system (Wootz et al., 2006). This event is as important as the activation of calpain, along with caspase-3, as revealed in our previous study (Milanovic et al., 2010). Several articles have reported the reduction of neuronal death after overexpression of XIAP resulting from the protective function of endogenous XIAP, which regulates caspase activation (Wang et al., 2004; Potts et al., 2003). It is probable that cellular caspase blockers provide a mechanism that safeguards against the unwanted induction of cell death, in particular, in long-living cells such as neurons. This is supported by the observation that proapoptotic proteins are expressed at high levels in the developing brain compared with adults (Korhonen et al., 2001; Yousefi et al., 2003). On the other hand, neurons have a profound requirement for morphological remod-

eling, processes in which caspases regulate local changes in synapses and spines (Gilman and Mattson, 2002). Given their destructive potential, the caspases that contribute to sublethal morphogenic changes are probably subjected to stringent control mechanisms. Therefore, it is probable that XIAP plays an important role as a caspase regulator that accommodates axonal and dendritic processes to changing physiological requirements (Vutskits et al., 2005; Unsain et al., 2013). In agreement with this, we detected that propofol anesthesia significantly changed expression of few representative proteins known to mediate synaptic plasticity at presynaptic (GAP-43, synaptophisin, and synuclein), transsynaptic (N-cadherin), and postsynaptic (drebrin and MAP-2; article in preparation) levels. Notably, at least some of these proteins might be substrates of caspase-3 and/or calpain proteases (Zakharov and Mosevitsky, 2001; Fifre et al., 2006; Jang et al., 2009; Han et al., 2013). Subsequent changes in synaptic transmission and dendritic spine morphology might interfere with neuronal plasticity, circuitry organization, and functional connectivity in the developing brain.

The upregulation of XIAP protein is significant in light of several studies that have demonstrated the neuroprotective effects of several compounds, such as lithium (Straiko et al., 2009), erythropoietin (Zacharias et al., 2010), and melatonin (Yon et al., 2005), in neonatal anesthetized rats. The exact mechanism through which these
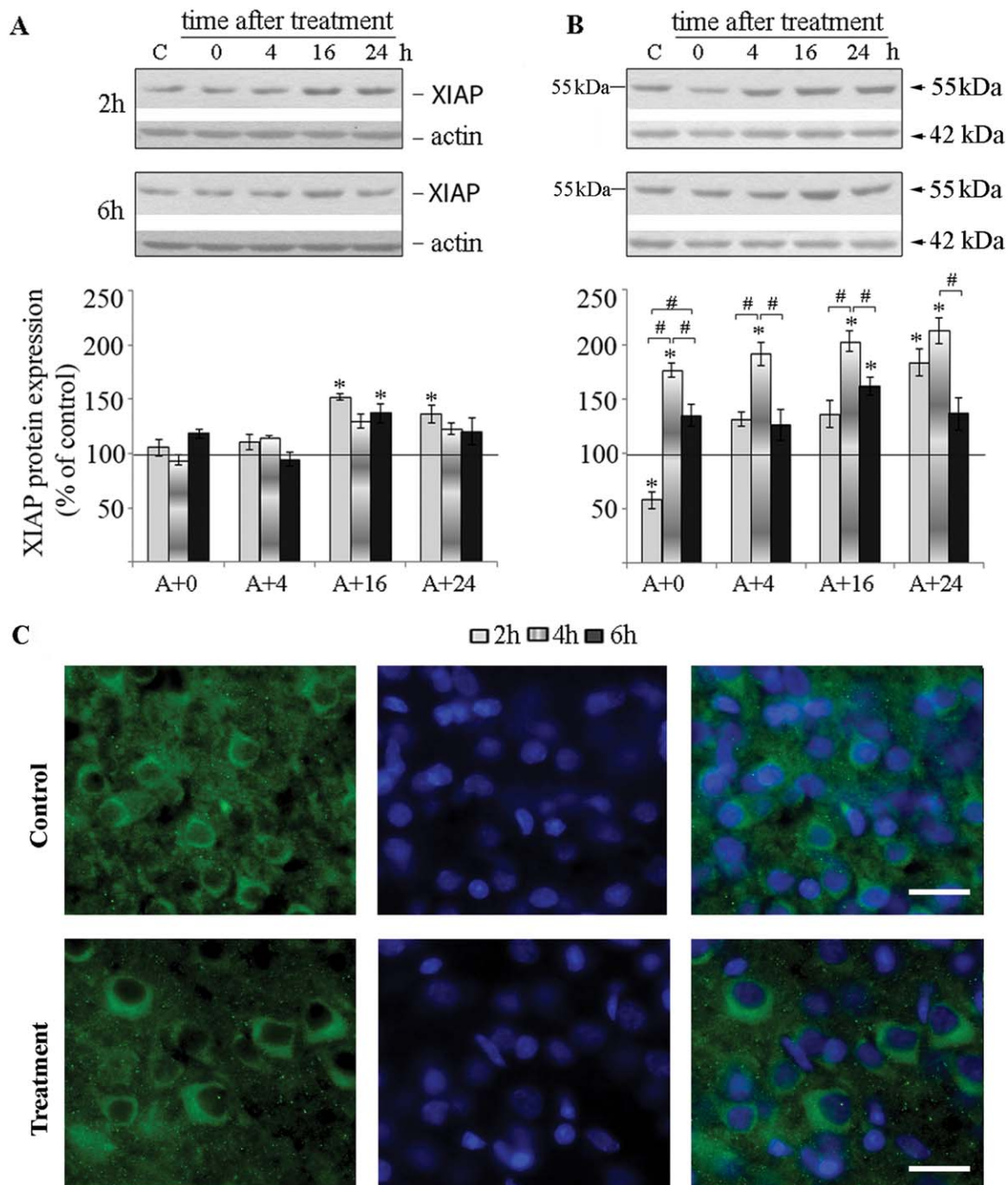
Fig. 7. Time course of XIAP protein expression in the cortex (**A**) and the thalamus (**B**) of P7 rats. The level of XIAP protein expression was established by Western blot analysis of total protein extracts. Each graph is accompanied by representative immnoblots obtained after 2- and 6-hr propofol anesthesia. **C:** Representative XIAP immunostaining of P7 rat brains at 24 hr time point after propofol exposure. XIAP immunoreactivity was localized predominantly in the perinuclear region in the thalamus of control animals; after propofol treatment, XIAP expression was stronger and diffused within the whole cell. Magnification, ×40. Results were normalized to the internal control (β-actin) and are expressed as mean ± SEM. ⋆$P < 0.05$ vs. the control value, presented as a black line; [#]$P < 0.05$ between treatments. Scale bars = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

substances protect against apoptotic cell death is not known. However, several new studies have provided evidence for the interaction between XIAP expression and melatonin, lithium, and erythropoietin, which were administered after brain injury (Sheth et al., 2009; Digicaylioglu et al., 2004; Li et al., 2010). This finding, together

with our results, implies that one of the potential strategies for preventing anesthesia–induced neuronal apoptosis in the developing brain could be based on mimicking endogenous antiapoptotic molecules such as XIAP.

In summary, the exposure of the neonatal rat brain to propofol anesthesia activates the extrinsic apoptotic pathway and expression of proneurotrophins in duration of exposure-, brain structure-, and temporal-specific manners during the critical period of synaptogenesis. Our results clearly show that propofol anesthesia concomitantly increased pAkt and XIAP expression. This increase establishes a fine balance between death and survival signals that serves to inhibit or to postpone cell death. Additional studies are required to clarify the details of the molecular mechanisms that underlie the toxicity of anesthetics. We believe that, to protect neurons from the deleterious effects of anesthesia during the early phase of brain development, an approach that would augment the innate prosurvival mechanisms in neurons by pharmacologically targeting pAKT and XIAP is justified.

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