Published in final edited form as:
J Neurochem. 2005 February ; 92(3): 701–704.

Anesthesia and post-mortem interval profoundly influence the regulatory serine phosphorylation of glycogen synthase kinase-3 in mouse brain

Xiaohua Li, **Ari B. Friedman**, **Myoung-Sun Roh**, and **Richard S. Jope**
Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham, Birmingham, Alabama, USA

Abstract

Glycogen synthase kinase-3 (GSK3) is a crucial enzyme contributing to the regulation of neuronal structure, plasticity and survival, is implicated as a contributory factor in prevalent diseases such as Alzheimer's disease and mood disorders and is regulated by a wide range of signaling systems and pharmacological agents. Therefore, factors regulating GSK3 in vivo are currently of much interest. GSK3 is inhibited by phosphorylation of serine-9 or serine-21 in GSK3β and GSK3α, respectively. This study found that accurate measurements of phospho-Ser-GSK3 in brain are confounded by a rapid post-mortem dephosphorylation, with ~90% dephosphorylation of both GSK3 isoforms occurring within 2 min post-mortem. Furthermore, three anesthetics, pentobarbital, halothane and chloral hydrate, each caused large in vivo increases in the serine phosphorylation of both GSK3β and GSK3α in several regions of mouse brain. Thus, studies of the phosphorylation state of GSK3 in brain, and perhaps in other tissues, need to take into account post-mortem changes and the effects of anesthetics and there is a direct correlation between anesthesia and high levels of serine-phosphorylated GSK3.

Keywords

anesthesia; glycogen synthase kinase-3; pentobarbital; post-mortem interval

Glycogen synthase kinase-3 (GSK3) has recently become recognized as an enzyme which influences many aspects of neuronal function, such as gene expression, architecture, plasticity and survival (Ali *et al*. 2001; Grimes and Jope 2001; Eldar-Finkelman 2002). These critical actions of GSK3 are mediated by the more than 40 known substrates of GSK3 (Jope and Johnson 2004). With this diverse array of functions and numerous substrates, the activity of GSK3 must be tightly controlled. The activities of the two isoforms of GSK3, GSK3α and GSK3β, are primarily regulated by phosphorylation of an N-terminal serine, serine-21 and serine-9, respectively, which inhibits activity. Several different kinases are capable of phosphorylating these regulatory serines on GSK3, including Akt (protein kinase B), protein kinase C, protein kinase A and others (Jope and Johnson 2004). Thus, many signaling systems converge on GSK3 to control its activity via serine phosphorylation, thereby contributing to the regulation of its impact on cellular functions.

Identified links between dysregulated GSK3 and diseases of the CNS have increased interest in the *in vivo* mechanisms which regulate GSK3 in the brain. GSK3 has been linked to the neuropathological hallmarks of Alzheimer's disease, amyloid plaques and neurofibrillary

Address correspondence and reprint requests to Dr Richard S. Jope, Department of Psychiatry and Behavioral Neurobiology, 1720 7th Avenue South, Sparks Center 1057, University of Alabama at Birmingham, Birmingham, AL 35294-0017, USA. E-mail: jope@uab.edu.

tangles, in numerous studies (Grimes and Jope 2001; Jope and Johnson 2004). Furthermore, GSK3 was linked to mood disorders by the discovery that the mood stabilizer lithium directly inhibits GSK3, raising the possibility that GSK3 may be inadequately controlled in mood disorders (Klein and Melton 1996). Recently, an association was identified between schizophrenia and an AKT1 haplotype associated with low Akt1 protein levels and reduced signaling to GSK3 (Emamian *et al*. 2004), lending support to previously identified links between schizophrenia and dysregulation of GSK3 (Kozlovsky *et al*. 2002).

The critical cellular actions of GSK3 and its links to several prevalent diseases of the CNS have recently led to *in vivo* investigations of the effects of a variety of pharmacological agents on the phosphorylation state of GSK3 in the brain. Changes in serine-phosphorylated GSK3 in animal brain have been identified after administration of lithium (De Sarno *et al*. 2002; Beaulieu *et al*. 2004), serotonergic agents (Li *et al*. 2004), dopaminergic drugs (Gil *et al*. 2003; Beaulieu *et al*. 2004; Emamian *et al*. 2004) and psychotomimetics (Svenningsson *et al*. 2003). Due to its links to several neurological and psychiatric diseases, there is also much interest in examinations of the phosphorylation state and activity of GSK3 in post-mortem brain from subjects with these conditions, especially Alzheimer's disease. However, in both animal and human studies, the post-mortem stability of the serine phosphorylation of GSK3 has not been examined and neither have the effects of anesthetics that are commonly used in animal experiments been investigated. Therefore, we investigated these parameters and report here that brain phospho-Ser-GSK3 is rapidly dephosphorylated post-mortem and phospho-Ser-GSK3 levels are greatly increased by several anesthetics. These findings both impact on the methods that can be used to study GSK3 in brain and reveal, for the first time, that GSK3 is regulated by anesthesia in mammalian brain.

Materials and methods

Adult male C57BL/6 mice (8–12 weeks old; Frederick Cancer Research, Frederick, MD, USA) were used for all experiments. Where indicated, mice were injected intraperitoneally with pentobarbital (100 mg/kg) or chloral hydrate (600 mg/kg), or exposed to vapors of halothane, and were maintained under a heat lamp. Anesthesia was monitored with the righting reflex by testing the ability of a mouse to right itself within 30 s of being placed on its back.

Mice were decapitated and brains rapidly dissected in ice-cold saline. Brain regions were homogenized in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 5 μg/mL pepstatin, 0.1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate and 100 nM okadaic acid. The lysates were centrifuged at 20 800 *g* for 10 min to remove insoluble debris. Protein concentrations in the supernatant fluids were determined using the Bradford protein assay (Bradford 1976).

Lysates were mixed with Laemmli sample buffer (2% sodium dodecyl sulphate) and placed in a boiling water bath for 5 min. Proteins were resolved in 10% sodium dodecyl sulphate–polyacrylamide gels, transferred to nitrocellulose and probed with antibodies to phospho-Ser9-GSK3β, phospho-Ser21-GSK3α, phospho-Tyr276/216-GSK3α/β or total GSK3β, GSK3α or GSK3α/β (Cell Signaling Technology, Beverly, MA, USA). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG, followed by detection with enhanced chemiluminescence, and the protein bands were quantitated with a densitometer.

Results

The post-mortem stability of the serine phosphorylation of GSK3 was examined by incubating tissue at room temperature (22°C) for 0–30 min after decapitation before removing the brain from the skull. Immunoblots of phospho-Ser9-GSK3β and phospho-Ser21-GSK3α of samples from the cerebral cortex showed that, although the total levels of GSK3β and GSK3α were stable for 30 min post-mortem, the regulatory serines of both GSK3 isoforms were rapidly dephosphorylated (Fig. 1a). Within just 10 min post-mortem, approximately 95% of the phosphorylated serine was dephosphorylated with each isoform of GSK3 (phospho-Ser9-GSK3β was 3 ± 1% of control levels 10 min postmortem; mean ± SEM; $n = 3$). In contrast to serine phosphorylation, no changes were detected in the tyrosine phosphorylation of either GSK3 isoform.

Several different types of anesthetic agents were used to test whether anesthesia altered the serine phosphorylation of GSK3. Mice were administered anesthetizing doses of three commonly used anesthetics, pentobarbital, halothane and chloral hydrate. Treatment with pentobarbital caused large increases in the levels of phospho-Ser9-GSK3β and phospho-Ser21-GSK3α in both the hippocampus and cerebral cortex (Fig. 1b). In contrast, there were no changes in the total levels of either isoform of GSK3 following pentobarbital administration. Examination of the levels of phospho-Ser9-GSK3β and phospho-Ser21-GSK3α in the hippocampus and cerebral cortex also revealed large increases during anesthesia induced by halothane (Fig. 1c) or chloral hydrate (Fig. 1d) while no changes occurred in GSK3β or GSK3α levels. Anesthesia-induced increases in phospho-Ser9-GSK3β in the cerebral cortex amounted to 596 ± 19, 288 ± 79 and 444 ± 65% of control levels and in the hippocampus to 520 ± 13, 275 ± 85 and 314 ± 98% (means ± SEM; $n = 3$) after pentobarbital, halothane and chloral hydrate, respectively. Thus, anesthesia induced by each of these three agents is associated with large increases in serine phosphorylation of GSK3.

The anesthesia-associated increases in the levels of phospho-Ser-GSK3 could be due to reduced rates of the rapid post-mortem loss of phospho-Ser-GSK3 in anesthetized brain, resulting in apparent increases in serine phosphorylation of GSK3. This was tested by comparing the rate of post-mortem dephosphorylation of phospho-Ser-GSK3 in brains from unanesthetized mice and mice anesthetized with pentobarbital. In these experiments, the post-mortem interval examined was restricted to only the first 10 min because the majority of the dephosphorylation of GSK3 occurs during this time period. In unanesthetized mice, the hippocampal and cortical levels of phospho-Ser9-GSK3β declined rapidly, being reduced at 2 min post-mortem by 91 ± 4 and 94 ± 5% (means ± SEM; $n = 4$) in the cerebral cortex and hippocampus, respectively. Administration of pentobarbital caused a large increase in the level of phospho-Ser9-GSK3β, as shown in Fig. 1. Calculations of the rate of loss of the serine phosphorylation in samples from matched control and pentobarbital-treated mice and using exposures of immunoblots with equivalent intensities revealed that the rate of post-mortem dephosphorylation was identical in control and pentobarbital-treated cerebral cortex and hippocampus (Fig. 2). These results clarify that anesthesia increased the serine phosphorylation of GSK3 and that this was not a result of a slowing of the post-mortem dephosphorylation of the regulatory serines.

The time-courses of the pentobarbital-induced increases in the levels of phospho-Ser-GSK3α/β and anesthesia were examined. During the first 4 h after pentobarbital administration, mice were fully anesthetized and the levels of phospho-Ser9-GSK3β and phospho-Ser21-GSK3α remained constant at levels several fold above those in unanesthetized mice in four brain regions (Fig. 3). After recovery from anesthesia, 6 h after pentobarbital administration, the phospho-Ser-GSK3 levels had returned to, or below, control basal values.

Discussion

Glycogen synthase kinase-3 is emerging as a key enzyme which exerts significant influence on neuronal structure, plasticity and survival and alterations of GSK3 have been linked to several widespread diseases, including bipolar affective disorder, schizophrenia and Alzheimer's disease (Ali *et al*. 2001; Grimes and Jope 2001; Eldar-Finkelman 2002). However, only recently has information about the regulation of GSK3 in brain *in vivo* begun to be obtained. The two main findings of the present investigation have a direct impact on this field as further *in vivo* regulators of GSK3 are investigated because both post-mortem interval and anesthesia were found to cause very large changes in the regulatory serine phosphorylation of both isoforms of GSK3.

It is well known that phosphorylated proteins are subject to variable rates of dephosphorylation during the post-mortem interval (Conway and Routtenberg 1979; Tsuyama *et al*. 1987; Walaas *et al*. 1989; Jope *et al*. 1991; Matsuo *et al*. 1994). The present results reveal an especially rapid post-mortem serine dephosphorylation of GSK3. Thus, *in vivo* studies of the phosphorylation and activity of GSK3 must carefully control for post-mortem changes. Furthermore, this finding indicates that measurements of GSK3 phosphorylation and activity in post-mortem human brain cannot provide a reliable indicator of its pre-mortem state.

Most animal experiments employ anesthetic agents before examinations of brain enzyme phosphorylation states and activities. This is especially true of immunocytochemical studies which often involve perfusion of animals during anesthesia to fix the brain. The present results demonstrate that, in such conditions, the serine phosphorylation state of GSK3 is markedly increased by anesthesia.

In conclusion, these findings have a significant impact on investigations of the *in vivo* effects of drugs or treatments on GSK3 in rodent brain and on studies of GSK3 in post-mortem human tissue. In both of these, rapid post-mortem dephosphorylation of GSK3 has obvious consequences; such effects must be minimized in animal experiments and such changes complicate the attainment of meaningful measurements in postmortem human tissue. In rodents the large effects of anesthetics indicate that these are likely to obfuscate treatment protocols which combine behavioral or drug treatment regimens with the use of these anesthetics. We also note that, until proven otherwise, it is possible that both postmortem delay and anesthetic agents may influence measurements of the phosphorylation state and activity of GSK3 in tissues other than the brain. Thus, these findings define conditions that are necessary to achieve meaningful measurements of the phosphorylation state and activity of GSK3 concerning both anesthesia and post-mortem protocols. Furthermore, this study revealed that serine phosphorylation of GSK3 is dramatically increased in anesthetized mammalian brain.

Acknowledgements

This work was supported by grants from the National Institutes of Health.

References

Ali A, Hoeflich KP, Woodgett JR. Glycogen synthase kinase-3: properties, functions, and regulation. Chem Rev 2001;101:2527–2540. [PubMed: 11749387]

Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR, Caron MG. Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. Proc Natl Acad Sci USA 2004;101:5099–5104. [PubMed: 15044694]

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. Anal Biochem 1976;41:248–254. [PubMed: 942051]

Conway RG, Routtenberg A. Endogenous phosphorylation in vitro: differential effects of brain state (anesthesia, post-mortem) on electrophoretically separated brain proteins. Brain Res 1979;170:313–324. [PubMed: 223723]

De Sarno P, Li X, Jope RS. Regulation of Akt and glycogen synthase kinase-3β phosphorylation by sodium valproate and lithium. Neuropharmacology 2002;43:1158–1164. [PubMed: 12504922]

Eldar-Finkelman H. Glycogen synthase kinase 3: an emerging therapeutic target. Trends Mol Med 2002;8:126–132. [PubMed: 11879773]

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA. Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia. Nat Genet 2004;36:131–137. [PubMed: 14745448]

Gil M, Zhen X, Friedman E. Prenatal cocaine exposure alters glycogen synthase kinase-3β (GSK3β) pathway in select rabbit brain areas. Neurosci Lett 2003;349:143–146. [PubMed: 12951189]

Grimes CA, Jope RS. The multi-faceted roles of glycogen synthase kinase-3β in cellular signaling. Prog Neurobiol 2001;65:391–426. [PubMed: 11527574]

Jope RS, Johnson GVW. The glamour and gloom of glycogen synthase kinase-3 (GSK3). Trends Biochem Sci 2004;29:95–102. [PubMed: 15102436]

Jope RS, Johnson GVW, Baird MS. Seizure-induced protein tyrosine phosphorylation in rat brain regions. Epilepsia 1991;32:755–760. [PubMed: 1720736]

Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA 1996;93:8455–8459. [PubMed: 8710892]

Kozlovsky N, Belmaker RH, Agam G. GSK-3 and the neurodevelopmental hypothesis of schizophrenia. Eur Neuropsychopharmacol 2002;12:13–25. [PubMed: 11788236]

Li X, Zhu W, Roh MS, Friedman AB, Rosborough K, Jope RS. In vivo regulation of glycogen synthase kinase-3β (GSK3β) by serotonergic activity in mouse brain. Neuropsychopharmacology 2004;29:1426–1431. [PubMed: 15039769]

Matsuo ES, Shin RW, Van Billingsley ML, deVoorde A, O'Connor M, Trojanowski JQ, Lee VM. Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. Neuron 1994;13:989–1002. [PubMed: 7946342]

Svenningsson P, Tzavara ET, Carruthers R, Rachleff I, Wattler S, Nehls M, McKinzie DL, Fienberg AA, Nomikos GG, Greengard P. Diverse psychotomimetics act through a common signaling pathway. Science 2003;302:1412–1415. [PubMed: 14631045]

Tsuyama S, Terayama Y, Matsuyama S. Numerous phosphates of microtubule-associated protein 2 in living rat brain. J Biol Chem 1987;262:10 886–10 892.

Walaas SI, Perdahl-Wallace E, Winblad B, Greengard P. Protein phosphorylation systems in postmortem human brain. J Mol Neurosci 1989;1:105–116. [PubMed: 2641277]

Abbreviations used

GSK3

glycogen synthase kinase-3

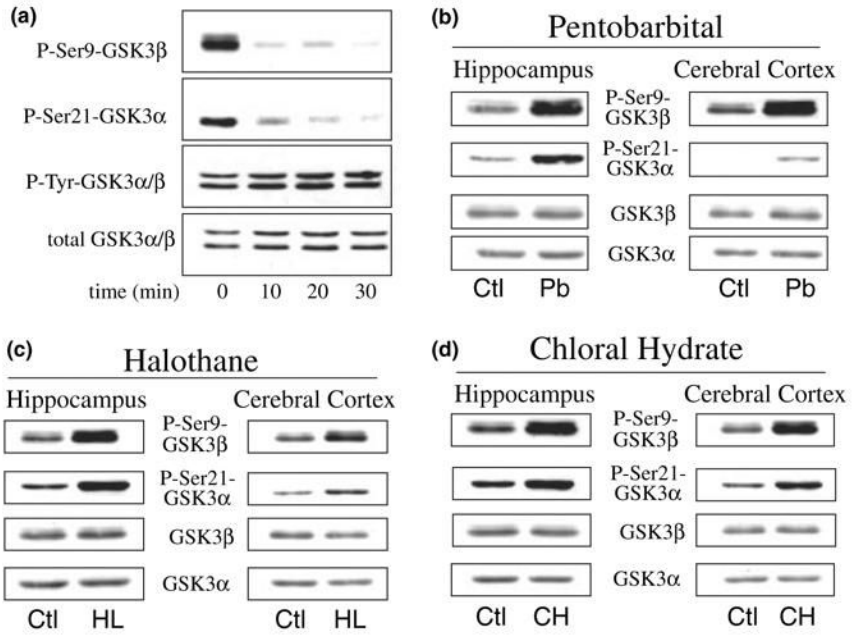

Fig. 1.

Phospho-Ser-GSK3 is rapidly dephosphorylated post-mortem and is increased by anesthesia. (a) Mouse brains were incubated at room temperature (22°C) for 0, 10, 20 or 30 min post-mortem before rapid dissection and homogenization. Samples of the cerebral cortex were immunoblotted for phospho-Ser9-GSK3β, phospho-Ser21-GSK3α, phospho-Tyr-GSK3α/β and total GSK3α/β (upper band, GSK3α; lower band, GSK3β). Anesthesia was induced in mice by administration of (b) pentobarbital (Pb; 100 mg/kg; 15 min), (c) halothane (HL; 5 min) or (d) chloral hydrate (CH; 600 mg/kg; 2 min). Anesthetic agents were tested individually in different experiments with paired control mice (Ctl) which received no anesthetic. Hippocampal and cerebral cortical samples were immunoblotted for phospho-Ser9-GSK3β, phospho-Ser21-GSK3α, total GSK3β and total GSK3α.

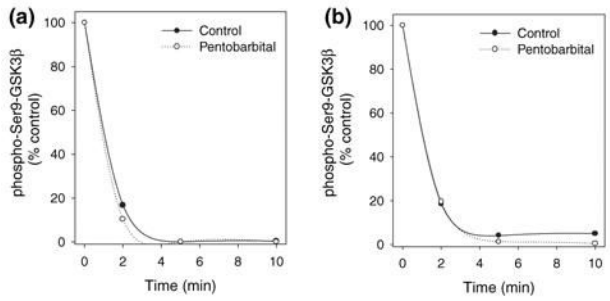

Fig. 2.

Similar post-mortem loss of phospho-Ser-GSK3 occurs in brains from control and pentobarbital-treated mice. (a) Hippocampus and (b) cerebral cortex of control and pentobarbital-anesthetized (100 mg/kg; 15 min) mice were extracted following post-mortem delays of 0, 2, 5 or 10 min. Samples were immunoblotted for phospho-Ser9-GSK3β and the loss of phosphorylation was calculated from densitometric measurements and presented as a percentage of the 0 min value of control or pentobarbital-treated mice.

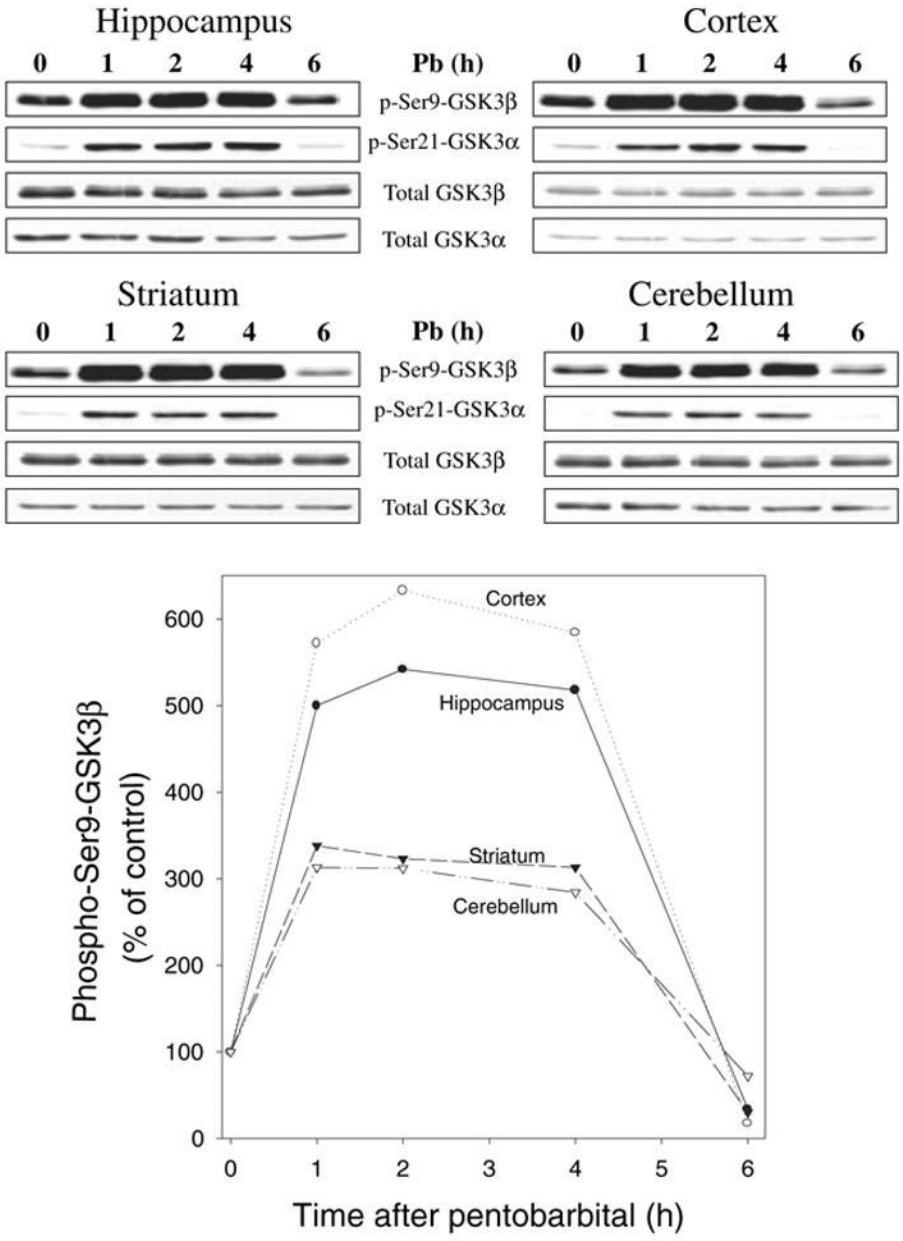

Fig. 3.

Time dependence of pentobarbital-induced increases in phospho-Ser-GSK3. The levels of phospho-Ser9-GSK3β, phospho-Ser21-GSK3α, total GSK3β and total GSK3α were measured in the hippocampus, cerebral cortex, striatum and cerebellum after administration of pentobarbital (Pb; 100 mg/kg) during anesthesia at 1, 2 or 4 h and after recovery from anesthesia 6 h after pentobarbital administration.