Toluene induced changes in excitatory amino acid metabolism during the sleep–wakefulness cycle

Guram G. Beradze,[a] Lali V. Shanshiashvili,[b,c] Isolda S. Chogovadze,[c] Irina V. Kalandadze,[c] Nikoloz T. Oniani,[c] Jeremy J. Ramsden[d,e] and David G. Mikeladze[c,*]

[a] *P. Shotadze Medical Academy, Tbilisi, Georgia*
[b] *Ilia State University, Tbilisi, Georgia*
[c] *I. Beritashvili Institute of Physiology, Tbilisi, Georgia*
[d] *Collegium Basilea, Institute of Advanced Study, Basel, Switzerland*
[e] *Cranfield University, Bedfordshire, England*

Many occupational studies have inferred central nervous system dysfunction as an outcome of toluene exposure. The sleep–wakefulness cycle is a universal model of brain integrative activity, which reflects multiple levels of the homeostatic processes. In this study, the structure of the sleep–wakefulness cycle of the rat, the quantitative ratio of phases, their electrographic characteristics and changes in the content of intraventricular glutamate, aspartate and arginine were determined in the normal state, after 10 days inhalation of toluene, 5 days after its withdrawal and after the action of chlorogenic acid, one of the most abundant polyphenols in the human diet with antioxidant, anticarcinogenic and neuroprotective properties. Using electroencephalography for the determination of behavioural state we found that toluene inhalation disrupts the normal sleep cycle, increased the total length of wakefulness, suppressed REM sleep, and reduced non-REM sleep. We have furthermore shown that the content of intraventricular glutamate was increased in all states of the sleep–wakefulness cycle while the concentration of aspartate was decreased. Arginine was diminished during wakefulness and non-REM sleep and increased in REM sleep. Intraperitoneal administration of chlorogenic acid resulted in rapid normalization both of the duration of the sleep–wakefulness cycle and the amino acid contents. These findings suggest that toluene engenders disturbance of excitatory amino acid and nitric oxide metabolism, which is reflected in the dynamics of the sleep–wakefulness cycle and duration of its phases.

Keywords: aspartate, arginine, chlorogenic acid, glutamate, rat

1. INTRODUCTION

According to evidence gathered by the World Health Organization [1], toluene is a surprisingly widely distributed toxic volatile substance. World production of toluene, once considered to be a harmless substitute for benzene, approaches 10 million tons annually and the spectrum of its distribution is very broad. Changes induced by toluene include acute and chronic neurotoxic effects and a variety of disorders that complicate the search for effective treatments for both acute and chronic toluene poisoning [2]. Given the widespread occurrence, both industrial and domestic, the study of the influence of this substance exceeds the bounds of the sphere of experimental and clinical interests and acquires social and demographic significance [3]. Toluene absorption by the human body occurs mainly via the respiratory tract. After inhalation of toluene in large doses it was found in different tissues and organs of laboratory animals, including the central nervous system, which is known to be especially sensitive to the influence of toluene [1]. In people with the habit of sniffing toluene, atrophy of different brain regions followed by different nervous and psychic disorders as well as by changes in the electrical activity of the brain has been observed [1]. Yet the unequivocal estimation of the neurotoxic effect of toluene from both clinical [2, 4] and experimental [5, 6] data is complicated because of biphasic (excitatory and inhibitory) effects conditioned by the variety of the dynamics of substance metabolism; it depends on toluene concentration, exposure duration and the presence of other accompanying factors. Hence, the necessity of pharmaco-kinetic modelling based on physiology and appropriately complex neurobiological investigations has become obvious [7].

* Corresponding author. E-mail: mikeladze@biphysiol.ge
Tel.: +99 532 37-47-24. Fax: +99 532 37-34-11.

Journal of Biological Physics and Chemistry **10** (2010) 96–101
Received 28 January 2010; *accepted* 30 September 2010

Changes in the ratio of sleep–wakefulness cycle phases have already been demonstrated, the character of which is conditioned by toluene concentration and the age of the experimental animals [8]. Work on this subject is scanty, although the sleep–wakefulness cycle appears to be a universal model for the characterization of homeostatic processes in the brain [9]. The neurochemical mechanism underlying the sleep–wakefulness cycle, its structural and electrographic changes, as well as qualitative and quantitative changes of its constituent phases and stages reliably and distinctly reflect the effects of various substances at different concentrations. Toluene has been reported to inhibit the NMDA receptors, probably through altering their subunit compositions [10, 11] and induce oxidative stress in several brain regions [12]. Toluene-induced diminution of Ca^{2+} signals linked to NMDA receptors could disrupt many aspects of central nervous system (CNS) function and thereby play an important part in CNS damage. Because the modulation of Ca^{2+} entry through NMDA receptors is an essential regulatory mechanism during the sleep–wakefulness cycle [13], toluene-induced alterations in NMDA-mediated Ca^{2+} signals with subsequent oxidative stress could disrupt normal sleep homeostasis.

Epidemiological evidence suggests that consumption of healthy foods containing phytochemical compounds might reduce the incidence of chronic degenerative diseases [14]. Chlorogenic acid, the ester of caffeic acid with quinic acid, is one of the most abundant polyphenols in the human diet. There is some evidence that chlorogenic acid has antioxidant and anxiolytic activities [15], causing a mild arousal effect [16], and that it attenuates behavioural deficits associated with strokes [17]. Since oxidative stress could change amino acid metabolism [18], we hypothesize that chlorogenic acid may normalize toluene-induced disruption in the sleep–wakefulness cycle by maintaining normal levels of excitatory amino acids in the brain. Starting from already available data, including those from our own previous investigations [19], the aim of the present research was to study the effect of toluene on the main characteristics of the structure and dynamics of the sleep–wakefulness cycle, and simultaneously quantify changes in the levels of the excitatory amino acids glutamate and aspartate as well as the nitric oxide (NO)-donating amino acid arginine. Furthermore, the protective effects of chlorogenic acid were examined.

2. METHODS

Experiments were carried out on 20 adult male Wistar rats (body weight 250–300 g) exposed to toluene during a period of 10 days. During preliminary surgery under anaesthesia (equitensine, 3 mL/kg) stainless steel standard electrodes were bilaterally implanted into the brain and cervical and oculomotor muscles, while into the lateral ventricle of the brain a special microcannula (Push-Pull Cannula C312GP, inner diameter 0.5 mm) was placed using a conventional method [20] in order to be able to collect intracranial fluid samples. Coördinates were selected according to a published stereotaxic atlas of the rat brain [21]. After a postoperation period (seven to ten days) continuous recording of animal sleep–wakefulness cycles was carried out daily from 10 am till 6 pm. The animals were kept in a special chamber that allowed them to move freely, and the recording of the sleep–wakefulness cycles as well as the collection of the required fluids was possible regardless of the state of sleep or wakefulness.[1]

Both the dynamics of the structure of the sleep–wakefulness cycle, the quantitative ratio of the phases, their electrographic and behavioural characteristics, and quantitative changes in amino acid concentrations were evaluated during preliminary background recording (3 days), during 10 days' inhalation of toluene at a concentration of 600 ppm in air and after cessation of toluene inhalation (5 days), or after intraperitoneal administration of chlorogenic acid (Sigma Chemical Co.) at a dose of 20 mg/kg.

Feature identification of 5–10 s intervals of the records was made according to relevant electrographic criteria: the states of wakefulness, non-REM sleep and REM sleep were determined.

For the analysis of amino acids from the lateral ventricle of the brain, the required 10 μL samples of intracranial fluid were collected by means of the microcannula. An internal standard (2 μmol/L of norleucine) was added to the sample as soon as the fluid was collected. Amino acid determination was carried out on a Pico Tag amino acid analysis system (Waters Associates, Milford, Mass.) according to the manufacturer's recommendation. Samples were derivatized with phenylisothiocyanate (PITC), and samples were then subjected to HPLC. Quantitative correction of glutamate, aspartate and arginine was carried out with regard to the presence of leucine, alanine

[1] All surgical and experimental procedures strictly adhered to the *Guide for the Care and Use of Laboratory Animals* (Washington, D.C.: National Academy of Sciences Press, 1996) and the Institutional Ethics Committee of the Beritashvili Institute of Physiology.

and tyrosine. The data were treated statistically using the ANOVA computer program. All results were expressed as mean ± SEM. Comparisons between groups were analysed by Student's *t* test. Differences were considered significant at $P < 0.05$.

3. RESULTS

3.1 Changes in the structure of the sleep–wakefulness cycle

We have found that toluene inhalation markedly disturbs the structure of the sleep–wakefulness cycle, as well as the electrographic characteristics of individual phases and the ratios of the quantitative distributions of the amino acids. *On the very first day of toluene inhalation*, toluene-induced changes were primarily expressed in the suppression of REM sleep, which occurred only 2 h after the beginning of the recording in the form of brief, rare and incomplete fragments. The duration of non-REM sleep was also disturbed, the length of separate episodes decreasing, although the frequency of occurrence of these short-lasting episodes increased; there was frequent alternation of wakefulness and non-REM sleep fragments. The electrographic characteristics of REM sleep were also changed: in particular, the characteristic prevailing rhythm of theta-range electrical activity of the brain was periodically substituted by slower delta-waves, while against a background of short-term atony of the cervical muscles single short-term discharges were often observed, accompanied by rapid saccadic eye movements ending in a startled waking up.

Toluene inhalation during the subsequent days of exposure changed the sleep–wakefulness cycle more significantly. The duration of wakefulness markedly increased and in some cases took 40–60% of the recording time (Fig. 1). During this time the animal was mainly quiet and changed its posture only during the frequent wakings from non-REM sleep or the very infrequent short-lasting REM sleep. However it quickly returned to the characteristic quiet state, during which short-lasting slow-wave activity of high amplitude developed periodically. It should be noted that such changes were largely maintained during the 5 days following the cessation of the 10 days' toluene inhalation.

Normalization of the sleep cycle deviations was rapidly induced under the influence of chlorogenic acid. In particular, on the first or second days after its administration, the sleep–wakefulness cycle as well as the amount and duration of the phase episodes approached the state characteristic of the norm (Fig. 1).

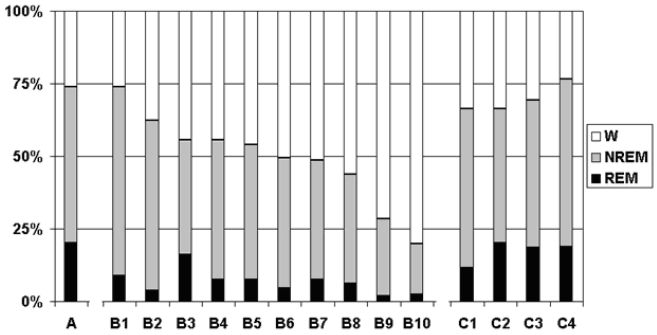

Figure 1. Ratios of the durations of sleep–wakefulness cycle phases (wakefulness (W), non-REM and REM sleep). The total duration of recording (10–18 h) is taken as 100%. A, the norm (average data from 3 days background recording); B1–B10, during the subsequent 1–10 days inhalation of toluene; C1–C4, after cessation of toluene inhalation and administration of chlorogenic acid during 1–4 days. Results are expressed as mean ± SEM of three independent experiments ($P < 0.05$).

3.2 Dynamics of amino acid concentration changes

Analysis of the amino acids in the intracranial fluid showed that the normal (i.e., in the absence of toluene) concentration of glutamate is the highest in non-REM sleep, while it significantly decreased during REM sleep, and especially during wakefulness. It is noteworthy that these dynamics were not changed under the influence of toluene (Fig. 2). However, a significant increase of the absolute amount of glutamate during the three states, exceeding the initial levels almost twofold, must be emphasized. After 10 days' exposure to toluene the amount of glutamate decreased under the influence of chlorogenic acid and returned to the normal level during wakefulness and REM sleep, but was significantly decreased in non-REM sleep (Fig. 2). Aspartate greatly decreased during all states of the sleep–wakefulness cycle, and especially significantly during non-REM sleep. Chlorogenic acid barely changed aspartate concentration in wakefulness episodes and non-REM sleep; in REM sleep it further decreased (Fig. 3). Under the influence of toluene the amount of arginine also changed significantly. The most obvious increase of its concentration was noted in REM sleep, and a great decrease was observed during wakefulness, and especially in non-REM sleep (Fig. 4).

Chlorogenic acid, which promoted the recovery of the initial control level of glutamate during wakefulness and REM (the decrease of glutamate in non-REM sleep is barely significant) was practically ineffective for normalizing aspartate, and had a quite different action with arginine: although the arginine concentration returned to slightly above its normal level during wakefulness, during fragments of REM and especially of non-REM sleep it was greatly decreased compared even to the norm.

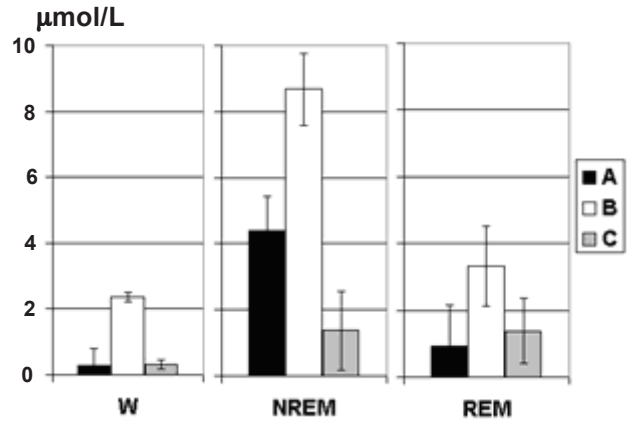

Figure 2. Dynamics of glutamate content. Changes in glutamate content (µmol/L) during wakefulness (W), non-REM and REM sleep in the norm (A), during toluene inhalation (B) and after chlorogenic acid administration (C). Results expressed as mean ± SEM of three independent experiments ($P < 0.05$).

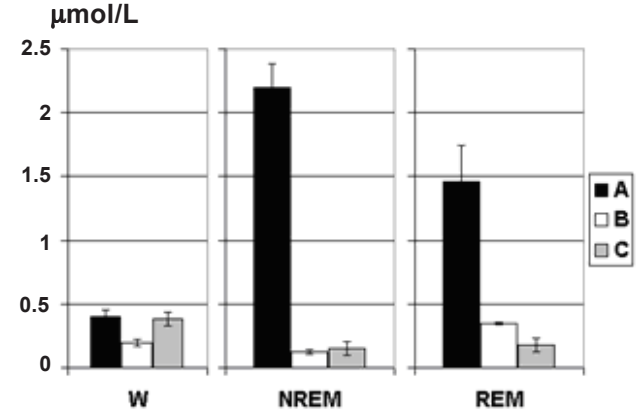

Figure 3. Dynamics of aspartate content. Changes in aspartate content (µmol/L) during wakefulness (W), non-REM and REM sleep in the norm (A), during toluene inhalation (B) and after chlorogenic acid administration (C). Results expressed as mean ± SEM of three independent experiments ($P < 0.05$).

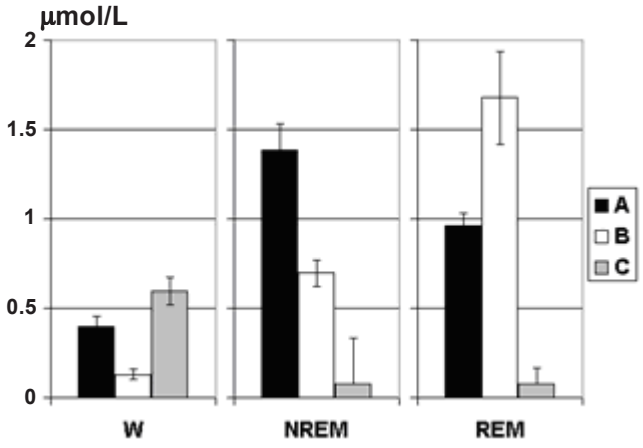

Figure 4. Dynamics of arginine content. Changes in arginine content (µmol/L) during wakefulness (W), non-REM and REM sleep in the norm (A), during toluene inhalation (B) and after chlorogenic acid administration (C). Results expressed as mean ± SEM of three independent experiments ($P < 0.05$).

4. DISCUSSION

There is evidence that a neurotoxic substance as potent as toluene, to which the nervous system is apparently especially sensitive, induces considerable chemical changes within various structures of the brain [22]. These changes include disturbance of the neurotransmission and modulating functions of biologically active compounds [23]. Understanding these changes is however complicated by the biphasic action of toluene on neurotransmitter systems [5]. In our experiments, toluene induced a significant increase of wakefulness duration, while a decrease of non-REM sleep, and even more strikingly of REM sleep, was observed. Concomitantly, a significant increase of glutamate in all the three states of sleep–wakefulness was noted; unexpectedly, since wakefulness and REM sleep are not neurochemically and functionally homogeneous with respect to their regulation; a significant importance of glutamatergic neurotransmission of the reticular formation in the regulation of REM sleep has been established [24]. Hence we propose that the relation of glutamate to other factors implicated in regulating wakefulness and REM sleep should be taken into account.

As shown by our previous investigations [19], glutamate and aspartate under normal conditions are quantitatively maximal during non-REM sleep, while the rate of formation of ammonia is decreased. In the same phase of sleep, arginine is also accumulated, indicating a low rate of its consumption and correspondingly inhibition of NO biosynthesis. On the other hand, states of REM sleep and wakefulness are characterized by a similar tendency, expressed by the intensive metabolism of glutamate, leading to a decrease of its concentration with an increase in the content of ammonia. During wakefulness and REM sleep phases arginine content also decreases, suggesting intensive formation of NO and probably induction of vasodilation.

Our experiments have shown that toluene changes the amino acid ratios. The concentration of glutamate in intracranial fluid increases during all three states (wakefulness, non-REM sleep and REM sleep). Under conditions of toluene poisoning a marked decrease of aspartate content takes place, which should attenuate the activation of NMDA receptors during wakefulness and REM sleep. There is also a quantitative decrease of arginine, resulting in a diminution of NO production. It is possible that the toluene-induced pronounced and prolonged increase of wakefulness might be due to an increase of glutamate, since its administration into the area of brain stem pedunculopontine tegmentum (PPT) sells, especially in high doses, evokes awakening of the animal to which it is administered [25]. Taking into

account that glutamatergic and cholinergic systems interact in modulating electrographic and behavioural arousal, our results imply that excitation of PPT cholinergic neurons must proceed from NMDA receptors, whose rate and duration of activation depend on the concentration of glutamate. The fact that NMDA micro-injection into a basal part of the rat forebrain prolongs wakefulness and suppresses non-REM sleep confirms this notion [25].

Taking into account data pointing to the similarity of REM sleep and active wakefulness and noting their competition [26], we propose that the prolongation of wakefulness has a compensatory character, and to some extent wakefulness takes up the function of REM sleep. The character of wakefulness is itself diverse, as shown by the variety of motor or behavioural idiosyncrasies, and also the emotional colouring that influences the transformation of REM sleep in a state of emotional tension to wakefulness. The threshold of wakefulness seems to be considerably decreased under the influence of toluene, disturbing development of REM sleep and the normal course of the cycle [9]. We propose that when under the influence of toluene during non-REM sleep, only glutamate increases, while aspartate and arginine decrease, and the increase of glutamate fails to initiate REM sleep, whose threshold exceeds that of wakefulness. On the other hand, the abundance of glutamate is quite enough to overcome the threshold of wakefulness and this state of affairs is reflected in the lengthening of wakefulness.

Within the framework of this scheme, it is pertinent to discuss the possibility of a neuroprotective effect of chlorogenic acid, the injection of which quickly restores the normal sleep–wakefulness cycle. At the same time, glutamate concentration significantly decreases in non-REM sleep (ending up even lower than the baseline), while aspartate increases a little, which may point to the intensification of glutamate transamination and correspondingly to the formation of aspartate. In wakefulness the arginine level is maintained within the normal limits under the influence of chlorogenic acid, while it markedly decreases during both non-REM and REM sleep. This points to the metabolism of arginine and the intensification of NO formation, which might cause modulation of the thalamo-cortical system that is considered to underlie the integral oscillatory mechanism.

ACKNOWLEDGMENTS

We thank Dr N. Skhirtladze and D. Sokhadze for their thoughtful comments on a draft of the manuscript. This study was supported by the Georgian National Scientific Fund (grant N 232).

REFERENCES

1. *Air Quality Guidelines*, 2nd edn. Copenhagen: World Health Organization Regional Office for Europe (2000).
2. Byrne, A., Kirby, B., Zibin, T. and Ensminger, S. Psychiatric and neurological effects of chronic solvent abuse. *Can. J. Psychiatry* **36** (1991) 735–738.
3. Mueser, K.T., Yarnold, P.R., Levinson, D.F., Singh, H., Bellack, A.S., Kee, K., Morrison, R.L. and Yadalam, K.G. Prevalence of substance abuse in schizophrenia: demographic and clinical correlates. *Schizophrenia Bull.* **16** (1990) 31–56.
4. Greenberg, M.M. The central nervous system and exposure to toluene: a risk characterization. *Environ. Res.* **71** (1997) 1–7.
5. Ikeuchi, Y., Hirai, H., Okada, Y., Mio, T. and Matsuda, T. Excitatory and inhibitory effects of toluene on neural activity in guinea pig hippocampal slices. *Neurosci. Lett*. **158** (1993) 63–66.
6. Stengard, K. Tail pinch increases acetylcholine release in rat striatum even after toluene exposure. *Pharmacol. Biochem. Behav.* **52** (1995) 261–264.
7. Urban, P. and Lukaš, E. The significance of neurophysiological methods in neurotoxicology. *Clin. Neurophysiol.* **112** (2001) 1139–1145.
8. Ghosh, T.K., Copeland, R.L. and Pradhan, S.N. Sensitivity of EEG in young rats to toluene exposure. *Pharmacol. Biochem. Behav.* **36** (1990) 779–785.
9. Oniani, T.N. *Integrative Function of the Limbic System*. Tbilisi: Metsniereba (1980).
10. Cruz, S.L., Mirshahi, T., Thomas, B., Balster, R.L. and Woodward, J.J. Effects of the abused solvent toluene on recombinant N-methyl-D-aspartate and non-N-methyl-D-aspartate receptors expressed in Xenopus oocytes. *J. Pharmacol. Exp. Ther.* **286 (**1998) 334–340.
11. Chen, H.H, Wei, C.T., Lin, Y.R., Chien, T.H. and Chan, M.H. Neonatal toluene exposure alters agonist and antagonist sensitivity and NR2B subunit expression of NMDA receptors in cultured cerebellar granule neurons. *Toxicol. Sci.* **85** (2005) 666–674.
12. Mattia, C.J., Ali, S.F. and Bondy, S.C. Toluene-induced oxidative stress in several brain regions and other organs. *Mol. Chem. Neuropathol.* **18** (1993) 313–328.
13. Prospero-Garcia, O., Criado, J.R. and Henriksen, S.J. Pharmacology of ethanol and glutamate antagonists on rodent sleep: a comparative study. *Pharmacol. Biochem. Behav*. **49** (1994) 413–416.
14. Tamimi, R.M, Lagiou, P., Adami, H.O., Trichopoulos, D. Prospects for chemoprevention of cancer. *J. Int. Med.* **251** (2002) 286–300.
15. Bouayed, J., Rammal, H., Dicko, A., Younos, C. and Soulimani, R. Chlorogenic acid, a polyphenol from *Prunus domestica* (mirabelle), with coupled anxiolytic and antioxidant effects. *J. Neurol. Sci.* **262** (2007) 77–84.
16. Shinomiya, K., Omichi, J., Ohnishi, R., Ito, H., Yoshida, T. and Kamei, C. Effects of chlorogenic acid and its metabolites on the sleep–wakefulness cycle in rats. *Eur. J. Pharmacol.* **504** (2004) 185–189.
17. Lapchak, P.A. The phenylpropanoid micronutrient chlorogenic acid improves clinical rating scores in rabbits following multiple infarct ischemic strokes: synergism

with tissue plasminogen activator. *Exp. Neurol.* **205** (2007) 407–413.

18. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. and Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39** (2007) 44–84.
19. Beradze, G., Shanshiashvili, L. and Mikeladze, D. The alteration of sleep–wakefulness phases can be induced by quantitative changes in excitatory amino acids. *Neurobiol. Sleep–Wakefulness Cycle* **1** (2001) 40–43.
20. Maddocks, S. and Setchell, B.P. Testosterone concentrations in testicular interstitial fluid collected with a push-pull cannula or by drip-collection from adult rats given testosterone or aminoglutethimide. *J. Endocrinol.* **121** (1989) 303–309.
21. Paxinos, G. and Watson, C. *The Rat Brain Stereotaxic Coordinates*. Sidney: Academic Press (1997).
22. Saavedra, H., De Marinis, A. and Palestini, M. Neuronal changes induced by chronic toluene exposure in the cat. *Arch. Ital. Biol.* **134** (1996) 217–225.
23. Castilla-Serna, I., Barragan-Mejia, M.G., Rodriguez-Perez, R.A., Garcia-Rillo, A. and Reyes-Vazquez, C. Effects of acute and chronic toluene inhalation on behavior, monoamine metabolism and specific binding (3H-serotonin and 3H-norepinephrine) of rat brain. *Arch. Med. Res.* **24** (1993) 169–176.
24. Jones, B.E. Arousal systems. *Frontiers Biosci.* **8** (2003) 438–451.
25. Datta, S., Patterson, E.H. and Spoley, E.E. Excitation of pedunculopontine tegmental NMDA receptors induces wakefulness and cortical activation in the rat. *J. Neurosci. Res.* **66** (2001) 109–116.
26. Maisuradze, L., Lortkipanidze, N., Eliozishvili, M., Oniani, N. and Oniani, T. A qualitative and quantitative assessment of the sleep–wakefulness cycle architecture in the post-paradoxical sleep deprivation period. *Sleep Research Online* **5** (2003) 59–66.