ELSEVIER

Biochemical Pharmacology

Biochemical Pharmacology 62 (2001) 401–405

Commentary

Neurotransmitters and apoptosis in the developing brain

Chrysanthy Ikonomidou[a,*], Petra Bittigau[a], Christian Koch[a], Kerstin Genz[a], Friederike Hoerster[a], Ursula Felderhoff-Mueser[b], Tatyana Tenkova[c], Krikor Dikranian[c], John W. Olney[c]

[a]*Department of Pediatric Neurology, Charité, Virchow Clinics, Humboldt University, Augustenburger Platz 1, 13353 Berlin, Germany*
[b]*Department of Neonatology, Charité, Virchow Clinics, Humboldt University, Augustenburger Platz 1, 13353 Berlin, Germany*
[c]*Department of Psychiatry, Washington University School of Medicine, 4940 Children's Place, St. Louis, MO 63110, USA*

accepted 10 May 2001

Abstract

In the immature mammalian brain during a period of rapid growth (brain growth spurt/synaptogenesis period), neuronal apoptosis can be triggered by the transient blockade of glutamate *N*-methyl-d-aspartate (NMDA) receptors, or the excessive activation of γ-aminobutyric acid ($GABA_A$) receptors. Apoptogenic agents include anesthetics (ketamine, nitrous oxide, isoflurane, propofol, halothane), anticonvulsants (benzodiazepines, barbiturates), and drugs of abuse (phencyclidine, ketamine, ethanol). In humans, the brain growth spurt period starts in the sixth month of pregnancy and extends to the third year after birth. Ethanol, which has both NMDA antagonist and $GABA_A$ agonist properties, is particularly effective in triggering widespread apoptotic neurodegeneration during this vulnerable period. Thus, maternal ingestion of ethanol during the third trimester of pregnancy can readily explain the dysmorphogenic changes in the fetal brain and consequent neurobehavioral disturbances that characterize the human fetal alcohol syndrome. In addition, there is basis for concern that agents used in pediatric and obstetrical medicine for purposes of sedation, anesthesia, and seizure management may cause apoptotic neuronal death in the developing human brain.

Keywords: Trauma; NMDA receptors; $GABA_A$ receptors; Synaptogenesis; Apoptosis; Ethanol; Phencyclidine; Ketamine; Barbiturates; Benzodiazepines; Anesthetics

1. Introduction

Apoptosis, or programmed cell death, also referred to as physiological cell death, takes place during normal development of the central nervous system. It is characterized by a sequence of very distinctive morphological changes in the dying neuron (recently reviewed by Ishimaru *et al.* [1] and Dikranian *et al.* [2]). In addition to recent advances in deciphering the molecular steps of apoptotic cell death in various *in vitro* systems [3], it is incumbent upon neuroscientists to develop a better understanding of mechanisms that regulate physiological apoptosis in the *in vivo* developing brain, since disruption of this physiological process may result in neurodevelopmental disorders. It is known from work with knockout mice that blocking various steps in the apoptotic cascade may cause severe migrational defects that are incompatible with life [4–6]. In addition, knockout experiments involving deletion of antiapoptotic genes cause mice to display severe neurological deficits and, in many cases, to die *in utero* or during the neonatal period [7,8]. It seems likely that for a full understanding of the many developmental neuropathology syndromes that occur in humans, it will be necessary to take into consideration both genetically determined disturbances and environmental factors that can influence the physiological cell death process. Consistent with this assumption, we have demonstrated recently that transient interference in the action of certain neurotransmitters during a critical stage in development, a stage in which trillions of synaptic connections are being formed, can trigger apoptotic degeneration of millions of neurons that otherwise would not have been deleted from the developing brain [9,10]. Here we will review evidence pertaining to this newly discovered mechanism and will discuss its relevance to human neurodevelopmental syndromes.

* Corresponding author. Tel.: +1-49-30-450-566273; fax: +1-49-30-450-566920.

E-mail address: hrissanthi.ikonomidou@charite.de (C. Ikonomidou).

Abbreviations: $GABA_A$, γ-aminobutyric acid; NMDA; *N*-methyl-D-aspartate; PCP; phencyclidine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

0006-2952/01/$ – see front matter
PII: PII S0006-2952(01)00696-7

2. Distinguishing apoptosis from other cell death processes

Wyllie and colleagues [8,11] proposed that all cell death processes can be classified as either apoptosis or necrosis, and much confusion has arisen as other researchers have attempted to grapple with this "either/or" classification system. The confusion is particularly great among neurobiologists because excitotoxicity, a cell death process to which most if not all CNS neurons are vulnerable, does not fit the Wyllie *et al.* definition of either apoptosis or necrosis. One source of confusion is that both apoptosis and excitotoxicity are processes that can lead to cell death, whereas necrosis refers to the state of being dead and to changes that occur after death. For this reason, we have proposed that neurobiologists should adopt a new frame of reference, one that is based on a more meaningful and intelligible dichotomy—"apoptotic versus excitotoxic" processes that can lead to cell death (necrosis). Another major source of confusion in this field stems from the widespread practice of relying on DNA fragmentation assays (gel electrophoresis "laddering" test, or TUNEL staining) to distinguish apoptosis from other cell death processes. As we and others have shown [1,8,11–15], it is treacherous to rely on such tests because they are not specific for apoptosis. Using both DNA fragmentation analysis and electron microscopy, we recently conducted a side-by-side comparison of a prototypic excitotoxic process, glutamate-induced neuronal death in the infant rat hypothalamus, and a prototypic apoptotic process, physiological cell death in the developing rodent brain [1]. We found that ultrastructurally the type and sequence of changes characterizing these two cell death processes are fundamentally very different, but DNA fragmentation tests did not distinguish between the two processes and were positive for both phenomena. Similar results were reported from several other laboratories. Thus, before concluding that a cell death process is apoptotic, we consider it important to subject the process to a detailed electron microscopic analysis.

3. Induction by NMDA antagonists of apoptotic neurodegeneration in the developing brain

Glutamate, acting at NMDA receptors, has trophic functions in the developing brain. Glutamate promotes proliferation and migration of neuronal progenitors and influences synaptic plasticity [16,17]. During the brain growth spurt period [18], NMDA receptors undergo a period of hypersensitivity that renders neurons bearing NMDA receptors exceedingly sensitive to excitotoxic degeneration [19,20]. During that exact same developmental period, which in the rat extends from late fetal life to about 2 weeks after birth, blockade of NMDA receptors for a period of hours triggers widespread apoptotic neurodegeneration in the brain [9].

This phenomenon was discovered while studying aspects of traumatic injury to the developing brain using a model of concussive head trauma in infant rats [21,22]. A concussive force applied to the skull overlying the parietal cortex of 7-day-old rats caused a small excitotoxic lesion at the impact site and a profound disseminated apoptotic response at distant sites that occurred in a delayed fashion. Administration of the NMDA antagonist dizocilpine (MK801) prior to concussive injury protected against the acute excitotoxic lesion at the impact site [21], but markedly enhanced the magnitude of the disseminated apoptotic response to trauma [22]. This finding raised the question of whether NMDA antagonists might promote physiological apoptosis, which occurs naturally in the developing brain. Indeed, we found that the NMDA antagonist MK801 when administered to 7-day-old infant rats triggers a massive apoptotic neurodegenerative response affecting many neurons in several major regions of the developing brain, as did the NMDA antagonists phencyclidine (PCP), ketamine (Fig. 1), and 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP) [9]. The time window of vulnerability to the neurotoxic effect of NMDA antagonists coincides with the period of synaptogenesis, which in the human spans the last 3 months of pregnancy and extends into the third year of life [18]. Different brain regions display different age-dependency profiles to the proapoptotic effect of NMDA receptor blockade, leading to different patterns of neuronal loss depending on the time of exposure [9]. Hence, depending on timing, this neurodevelopmental mechanism has the potential to produce a variety of neurological and behavioral deficits.

Given the fact that drugs of abuse, such as PCP, ketamine (Special K), and ethanol, as well as anesthetics (ketamine, nitrous oxide) used in pediatric medicine, block NMDA receptors, the question arises as to whether and under what circumstances such compounds may damage the human fetal, neonatal, and infant brain.

4. Induction by GABAergic agents of apoptotic neurodegeneration in the developing brain

In an attempt to study other possible mechanisms that regulate neuronal survival during brain development, we examined whether interference in the action of several other neurotransmitter systems might trigger apoptotic neurodegeneration. We found no appreciable apoptotic response to dopamine receptor agonists or antagonists, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)/kainate receptor antagonists, muscarinic cholinergic receptor antagonists, or blockers of voltage dependent calcium channels, but we did detect a striking apoptotic response to agents that act as agonists at $GABA_A$ receptors [10]. The benzodiazepines diazepam and clonazepam, and the barbiturates phenobarbital and pentobarbital, in a dose-dependent manner, triggered widespread apoptotic cell death in the infant rat brain. The distribution pattern of degeneration was similar for each GABAergic agent and differed in several respects

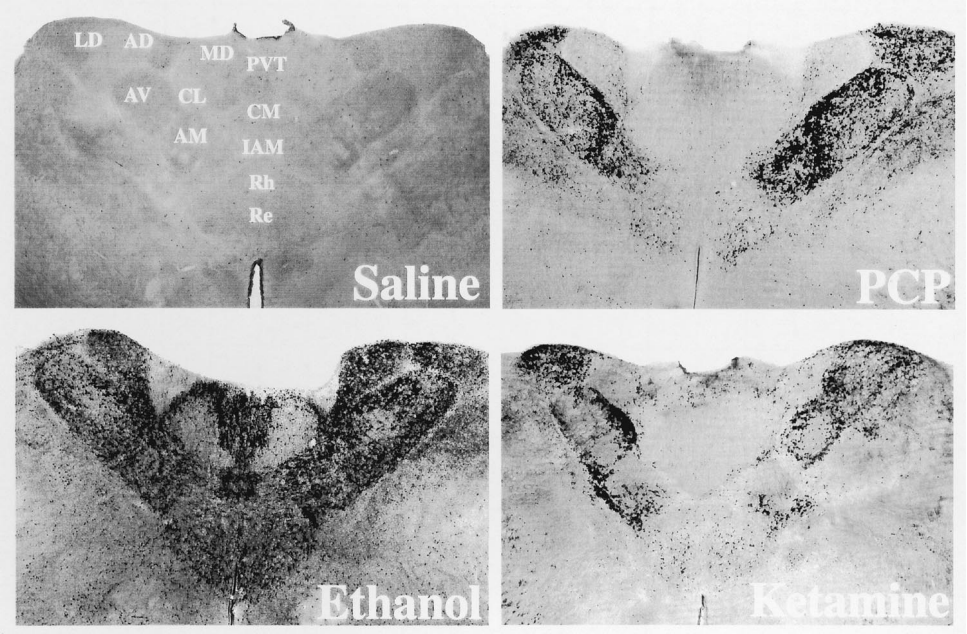

Fig. 1. Low magnification (×25) light microscopic overviews of silver-stained transverse sections from the thalamus of 8-day-old rats treated 24 hr previously with saline, phencyclidine (PCP), ethanol, or ketamine. Degenerating neurons (small dark dots) are abundantly present in several brain regions following PCP, ethanol, or ketamine but are only sparsely present following saline treatment. Note that PCP and ketamine both affect neurons in laterodorsal and ventral nuclei of the thalamus (LD, AV, AM), whereas the ethanol pattern also involves mediodorsal and ventromedial thalamic nuclei (AD, MD, CL, PVT, CM, IAM, Rh, Re). Abbreviations: laterodorsal (LD), anterodorsal (AD), anteroventral (AV), mediodorsal (MD), anteromedial (AM), paraventricular (PVT), central lateral (CL), central medial (CM), interanteriomedial (IAM), rhomboid (Rh), and reuniens (Re) thalamic nuclei. The ethanol pattern includes all nuclei, as would be expected if it acts by a combined action involving blockade of NMDA receptors plus activation of $GABA_A$ receptors [10].

from that induced by NMDA antagonists. Similar to the proapoptotic effect of NMDA antagonists, an apoptotic response to benzodiazepines and barbiturates could only be elicited during the brain growth spurt period in rats. Like the reaction to NMDA antagonists, vulnerability of different brain regions to GABAmimetics also changed with age, again raising the possibility that, depending upon the time of exposure to these agents, different patterns of neurodegeneration and potentially different neurobehavioral disturbances might occur.

5. Induction by ethanol of apoptotic neurodegeneration in the developing brain

Ethanol is, and has been for thousands of years, the most widely abused drug in the world. In the 1970s it was recognized that *in utero* ethanol exposure of the human fetus can result in a neurodevelopmental syndrome called fetal alcohol syndrome (FAS) or fetal alcohol effects (FAE) [23,24]. FAS is characterized by craniofacial anomalies, microcephaly, and mental retardation. FAS constitutes the more severe form of impairment due to intrauterine exposure to ethanol, whereas less severe cases, especially those primarily limited to neurobehavioral disturbances, are referred to as FAE [23–26].

The pathophysiology of the neurotoxic effects of ethanol remained unclear for decades. Evidence that ethanol has NMDA antagonist- [27–30] and $GABA_A$-agonist properties prompted us to evaluate its ability to mimic the proapoptotic effects of other NMDA antagonists and $GABA_A$ agonists. Administration of ethanol to 7-day-old infant rats triggered a very robust neurodegenerative response (Figs. 1 and 2) [10]. Maintaining blood ethanol concentrations at or above 200 mg/dL for 4 consecutive hr during the brain growth spurt period was required to trigger substantial neurodegeneration, and if ethanol concentrations remained above 200 mg/dL for more than 4 hr, the degenerative response became progressively more severe and more widespread in proportion to how long the concentrations remained above this level. Evaluation of the ethanol-induced degenerative response by electron microscopy revealed that it conforms to the criteria for apoptotic cell death [10].

Interestingly, superimposing the neurodegeneration patterns that resulted from treating infant rats with NMDA antagonists and $GABA_A$ agonists resulted in the neurodegeneration pattern caused by ethanol [10].

6. Neurotransmitters, apoptosis, and the developing human brain

Transient exposure of the mammalian brain to ethanol during the synaptogenesis period causes the death of millions of neurons. This finding provides a likely explanation for the reduced brain mass and neurobehavioral distur-

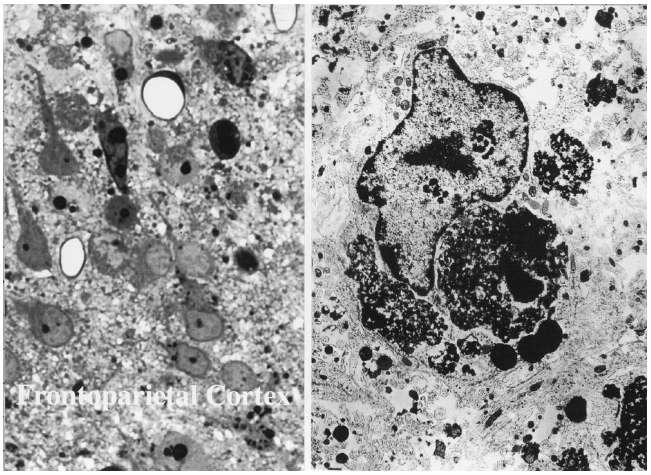

Fig. 2. Light micrographic (left panel) and electron micrographic (right panel) views taken from the frontoparietal cortex of an 8-day-old rat following treatment with ethanol on postnatal day 7. Degenerating neurons of different sizes are depicted in the plastic section on the left. They display nuclear pycnosis, chromatin fragmentation, and darkening of the cytoplasm, conforming with the diagnosis of apoptosis (methylene blue/azure II staining; magnification × 260). Shown in the electron micrograph are fragments of an apoptotic cell at a late stage (apoptotic bodies), which are being engulfed by a glial cell (magnification × 4800).

bances associated with the human FAE/FAS, in that the human synaptogenesis/brain growth spurt period includes the last 3 months of gestation [18]. The blood ethanol levels required to trigger apoptotic neurodegeneration in the immature rat brain (200 mg/dL lasting 4 hr or more) are in the range that a human fetus might be exposed to by maternal ingestion of a moderate to heavy dose of ethanol. Especially noteworthy is the fact that it only requires a single intoxication episode for the fetal brain damage to occur.

Ethanol provides a prime example of an agent that, by interfering with neurotransmitter systems, can quietly delete large numbers of neurons from the developing brain and give rise to neurobehavioral and psychiatric disturbances that may become manifest at various ages from infancy to adulthood. Since different neuronal populations in the fetal brain have different temporal patterns for responding to the apoptosis-inducing effects of ethanol, different combinations of neuronal groups will be deleted depending on the time of exposure, which explains why fetal ethanol exposure gives rise to a wide spectrum of neuropsychiatric disturbances [31]. Hyperactivity/attention deficit disorder, learning disabilities, and mental retardation have been associated with the FAS/FAE, as have adult-onset psychiatric problems, including psychosis and major depressive disorders [31].

NMDA antagonists and $GABA_A$ agonists are used as sedatives, anesthetics, tranquilizers, and anticonvulsants in obstetric and pediatric medicine [32]. In recent work we performed to determine apoptogenic threshold doses of barbiturates and benzodiazepines, we found that anticonvulsant doses are sufficient to trigger an apoptotic response in the developing rat brain [33]. Thus, long-term treatment of epileptic women with barbiturates and benzodiazepines late in pregnancy or children in the first years of life may entail the risk of deleting immature neurons by apoptosis. This mechanism can explain reduced head circumference and cognitive impairment in children of women with epilepsy and in humans who received treatment with phenobarbital during their first years of life [34,35].

In the context of pediatric anesthesia, combinations of NMDA antagonists (ketamine, nitrous oxide) and $GABA_A$ agonists (propofol, isoflurane, benzodiazepines, barbiturates) are used for prolonged periods at doses that impair consciousness, and thus produce a degree of intoxication comparable to that associated with prolonged high blood levels of ethanol. Further studies will be necessary to determine to what extent established anesthetic practices may pose significant risk to the developing human brain.

7. Conclusions

In recent pharmacological studies, we have shown that the blockade of NMDA glutamate receptors or excessive activation of $GABA_A$ receptors during synaptogenesis triggers widespread apoptotic neurodegeneration in the developing rodent brain. Our findings have clinical significance in that the brain growth spurt in humans extends from the sixth month of gestation to three years after birth, and during this period immature humans are sometimes exposed to drugs that block NMDA receptors and/or are excessively active $GABA_A$ receptors. For example, drugs in both of these classes are sometimes abused by pregnant mothers, and are routinely used as sedatives, anticonvulsants, or anesthetics in obstetrical and pediatric medicine. Ethanol, because it has both NMDA antagonist and GABAmimetic properties, is particularly effective in triggering this type of neurodegenerative reaction, and this provides a likely explanation for the reduced brain mass and lifelong neurobehavioral disturbances resulting from intrauterine exposure of the human fetus to ethanol. Research is needed to explore the possibility that use of apoptogenic drugs in obstetrical and pediatric medicine can cause alcohol-like effects that have heretofore escaped detection or have been ascribed to unknown causes.

Acknowledgments

This work was supported by DFG Grant Ik2/2–1 and NIH Grants AG 11355, DA 05072, EY 08089, and HD 37100. J.W.O. is an NARSAD 2000 Toulmin Distinguished Investigator Awardee.

References

[1] Ishimaru MJ, Ikonomidou C, Tenkova TI, Der TC, Dikranian K, Sesma MA, Olney JW. Distinguishing excitotoxic from apoptotic

neurodegeneration in the developing rat brain. J Comp Neurol 1999; 408:461–76.
[2] Dikranian K, Ishimaru MJ, Tenkova T, Labruyere J, Qin YQ, Ikonomidou C, Olney JW. Apoptosis in the *in vivo* mammalian brain. Neurobiol Dis, in press.
[3] Hengartner MA. The biochemistry of apoptosis. Nature 2000;407: 770–6.
[4] Roth KA, Kuan C, Haydar TF, D'Sa-Eipper C, Shindler KS, Zheng TS, Kuida K, Flavell RA, Rakic P. Epistatic and independent function of caspase-3 and Bcl-X_L in developmental programmed cell death. Proc Natl Acad Sci USA 2000;97:466–71.
[5] Yoshida H, Kong Y-Y, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM, Mak TW. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 1998;94:739–50.
[6] Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA. Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. Cell 1998;94: 325–37.
[7] Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev 2000;14:23–7.
[8] Wyllie AH, Kerr JF, Currie AR. Cell death in the normal neonatal rat adrenal cortex. J Pathol 1973;111:255–61.
[9] Ikonomidou C, Bosch F, Miksa M, Vöckler J, Bittigau P, Pohl D, Dikranian K, Tenkova T, Turski L, Olney JW. Blockade of glutamate receptors triggers apoptotic neurodegeneration in the developing brain. Science 1999;283:70–4.
[10] Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Hörster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 2000;287:1056–60.
[11] Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972;26:239–57.
[12] Gwag BJ, Koh JY, Demaro JA, Ying HS, Jacquin M, Choi DW. Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. Neuroscience 1997;77:393–401.
[13] Charriaut-Marlangue C, Ben-Ari Y. A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport 1995;7:61–4.
[14] Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995;21:1465–8.
[15] Collins RJ, Harmon BV, Gobé GC, Kerr JFR. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. Int J Radiat Biol 1992;61:451–3.
[16] Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. Science 1993;260:95–7.
[17] Guerrini L, Blasi F, Denis-Donini S. Synaptic activation of NF-κB by glutamate in cerebellar granule neurons *in vitro*. Proc Natl Acad Sci USA 1995;92:9077–81.
[18] Dobbing J, Sands J. The brain growth spurt in various mammalian species. Early Hum Dev 1979;3:79–84.
[19] McDonald JW, Silverstein FS, Johnston MV. Neurotoxicity of *N*-methyl-d-aspartate is markedly enhanced in developing rat nervous system. Brain Res 1988;459:200–3.
[20] Ikonomidou C, Mosinger JL, Salles KS, Labruyere J, Olney JW. Sensitivity of the developing rat brain to hypobaric/ischemic damage parallels sensitivity to *N*-methyl-aspartate neurotoxicity. J Neurosci 1989;9:2809–18.
[21] Ikonomidou C, Qin Y, Labruyere J, Kirby C, Olney JW. Prevention of trauma-induced neurodegeneration in infant rat brain. Pediatr Res 1996;39:1020–7.
[22] Pohl D, Bittigau P, Ishimaru MJ, Stadthaus D, Fuhr S, Voeckler J, Huebner C, Olney JW, Ikonomidou C. Apoptotic cell death triggered by head injury in infant rats is potentiated by NMDA antagonists. Proc Natl Acad Sci USA 1999;96:2508–13.
[23] Jones KL, Smith DW, Ulleland CN, Streissguth AP. Pattern of malformation in offspring of chronic alcoholic mothers. Lancet 1973;i: 1267–71.
[24] Clarren SK, Alvord AC, Sumi SM, Streissguth AP, Smith DW. Brain malformations related to prenatal exposure to ethanol. J Pediatr 1978; 92:64–7.
[25] Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. Lancet 1973;ii:999–1001.
[26] Sulik KK, Johnston MC, Webb MA. Fetal alcohol syndrome: embryogenesis in a mouse model. Science 1981;214:936–8.
[27] Lovinger DM, White G. Ethanol potentiation of 5-hydroxytryptamine receptor-mediated ion current in neuroblastoma cells and isolated adult mammalian cells. Mol Pharmacol 1991;40:263–70.
[28] Hoffman PL, Rabe CS, Moses F, Tabakoff B. *N*-Methyl-d-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. J Neurochem 1989;52:1937–40.
[29] White G, Lovinger DM, Weight FF. Ethanol inhibits NMDA-activated current but does not alter GABA-activated current in an isolated adult mammalian neuron. Brain Res 1990;507:332–6.
[30] Chandler LJ, Guzman NJ, Sumners C, Crews FT. Magnesium and zinc potentiate ethanol inhibition of NMDA-stimulated nitric oxide synthase in cortical neurons. J Pharmacol Exp Ther 1994;271:67–75.
[31] Famy C, Streissguth AP, Unis AS. Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects. Am J Psychiatry 1998;155: 552–4.
[32] Jevtovic-Todorovic V, Todorovic SM, Mennerick S, Powell S, Dikranian K, Benshoff N, Zorumski CF, Olney JW. Nitrous oxide (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. Nat Med 1998;4:460–3.
[33] Bittigau P, Genz K, v. Engelbrechten S, Hoerster F, Dikranian K, Olney JW, Ikonomidou C. Antiepileptics which enhance GABAergic inhibition cause neuronal apoptosis in the developing CNS. Soc Neurosci Abstr 2000;26:323.
[34] Zahn CA, Morrell MJ, Collins SD, Labiner DM, Yerby MS. Management issues for women with epilepsy. Neurology 1998;51:949–56.
[35] Dessens AB, Cohen-Kettenis PT, Mellenbergh GJ, Koppe JG, van de Poll NE, Boer K. Association of prenatal phenobarbital and phenytoin exposure with small head size and with learning problems. Acta Paediatr 2000;89:533–41.