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Epileptogenesis in diacylglycerol kinase epsilon deficiency up-regulates COX-2 and tyrosine hydroxylase in hippocampus

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Abstract

Diacylglycerol kinase (DGK) phosphorylates the second messenger diacylglycerol (DAG) to yield phosphatidic acid, two neural signaling elements that function to modulate synaptic activity. Of the nine mammalian DGK isotypes known, DGK epsilon (DGKε) shows specificity for arachidonoyldiacylglycerol (20:4-DAG) and selectively contributes to modulate brain signaling pathways linked to synaptic activity and epileptic seizure activity. In this study, we examined changes in gene transcription in a mouse kindling model of epileptogenesis using control DGKε (+/+) and DGKε-knockout (−/−) mice. Total RNA was isolated from the hippocampus and analyzed using RNA and DNA arrays. Significantly altered gene-expression levels were confirmed independently using Western immunoblot analysis. In agreement with our previous studies, a very few number of genes reached a significance of twofold or greater (either up- or down-regulated; $p < 0.05$). Among the most significantly up-regulated genes in DGKε (+/+) mice included those encoding the inducible prostaglandin synthase cyclooxygenase-2 (COX-2) and tyrosine hydroxylase (TH), also known as tyrosine 3-monooxygenase, the rate-limiting enzyme of catecholamine biosynthesis. Kindled DGKε (−/−) animals exhibited no large increases in COX-2 or TH gene expression. These data, plus our previous findings that DGKε (−/−) mice show higher resistance to electroconvulsive shock, suggest an interplay between, and a regulatory role for, DGKε, COX-2, and catecholamine signaling during kindling epileptogenesis.

Keywords: Diacylglycerol kinase epsilon; Epilepsy; Gene expression; Cyclooxygenase-2; Tyrosine hydroxylase; Kindling epileptogenesis

The second messenger arachidonic acid (20:4) and inositol lipid-derived diacylglycerol (DAG), two highly bioactive lipids, have been implicated in epileptogenesis [1,2]. Enzymes responsible for inositol lipid biosynthesis may use 20:4 lipids as substrates; DAG kinase epsilon (DGKε), a member of the DGK gene family, selectively phosphorylates 20:4-DAG, generating phosphatidic acid. DGKε thereby regulates neural signal transduction by modulating the balance between the two abundant signaling lipids DAG and phosphatidic acid. Targeted deletion of the DGKε gene in DGKε (−/−) mice has been an effective approach to assess the contribution of DGKε to arachidonate enrichment and seizure response [2]. The following experiments were undertaken to identify gene-expression changes that accompany kindling epileptogenesis in DGKε (−/−)-knockout and control DGKε (+/+) mice. Surprisingly, very few large changes in gene expression were observed. Among the most significantly up-regulated changes in gene expression in DGKε-knockout (−/−) mice included those encoding the neuronal-enriched inducible prostaglandin synthase cyclooxygenase-2 COX-2 [3–7], and tyrosine 3-monooxygenase (also known as tyrosine hydroxylase, TH), which catalyzes the first committed step in catecholamine synthesis [8–11]. These findings further support the idea that elements derived from 20:4-inositol lipid signaling are involved in the basic genetic responses to kindling epilepsy.

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Methods

Experimental design. Two-month-old control DGKε (+/+) or DGKε-knockout (−/−) mice were implanted with electrode units (Tripolar, Plastic One, Roanoke, VA) in the right dorsal hippocampus; a ground electrode was attached to the occipital bone. At 10 days postsurgery, kindling was accomplished after sub-convulsive electrical stimulation (6 times daily for 4 days, using a 10-s train of 50-Hz biphasic pulses of 75–100-μA amplitude). Seizures were graded according to the Racine Scale. Animals were killed, and total RNA and proteins were co-isolated after the last stimulation of the fourth day.

RNA isolation and quality control. Left and right hippocampal total RNA was isolated using Trizol reagent (Invitrogen Life Sciences, Carlsbad, CA) and RNA quality was screened and quality-controlled using RNA LabChips (Caliper Technologies, Mountain View, CA). Each total RNA sample was scanned from 220 to 320 nm using a GeneSpec III spectrophotometer (Hitachi Genetics, Alameda, CA) and the $A_{260/280}$ of total RNA (based on peak area) was typically >1.8. RNA samples were next analyzed for spectral purity and concentration (ng/μl) using RNA Nano LabChip analysis Chips and a 2100 Bioanalyzer (Caliper Technologies, Mountainview, CA; Agilent Technologies, Palo Alto, CA) [11]. Ratios of 28S/18S all exceeded 1.4; total RNA samples not meeting the strict specifications of an $A_{260/280}$ of >1.8 or 28S/18S ratios >1.4 were not used in these experiments. Poly(A)$^+$ messenger RNA populations ranged in size from 0.2 to about 9 kb (data not shown). Importantly, no significant differences between the spectral purity, molecular size, or yield of total RNA between kindled animals and controls were noted [11].

RNA labeling and DNA-array analysis. Total RNA was labeled using the Enzo Bioarray High Yield RNA Transcript Labeling system (Affymetrix, Santa Clara, CA), hybridized to the Test3 and analytical GeneChips, washed, and scanned as previously described [12].

Western analysis. Protein concentrations were determined using a dotMETRIC microassay and bovine serum albumin (A7888; Sigma) as a standard; levels of up-regulated genes were confirmed using Western immunoblot analysis [11]. Total protein was extracted and analyzed by Western blots using COX-1, COX-2, COX-3, or TH antibodies (kindly supplied by Dr. D. Simmons, purchased from Alpha Diagnostics, San Antonio, TX, or Santa Cruz Biotechnology, Santa Cruz, CA) that were used as previously described [1,8,11]. COX-1, COX-2, and TH antibodies used were sc-1752, sc-1745, and sc-7847, respectively, as used according to the manufacturer's instructions (Santa Cruz). Importantly, human COX-3 antibodies were derived from N-terminal peptides corresponding to the first 13 amino acids of human COX-3 (MSRECDPGARWGC) [13]; COX-1 and COX-3 antibodies showed no cross-reactivity (Alpha Diagnostics, Cat. #COX-32A). Signal-intensity data were gathered by phosphorimaging onto molecular imaging screens (FUJIFILM) for 3–36 h using a Typhoon Molecular Imager system (Amersham Biosciences) as described previously [11–13].

Bioinformatics and statistical analysis. DNA-array data analysis and feature extraction were performed using GeneSpring 7.2 (Silicon Genetics, Redwood City, CA) analytical algorithms; Westerns were analyzed using the internal control of βTUBIII as previously described [11–13].

Results

Gene-expression patterns can be effectively displayed by plotting the data as 'volcano plot' (V-plot) representations (Fig. 1), in which all significantly up- and down-regulated genes are plotted according to their p value and fold-change in a scatter plot on a semi-log graph, using $p \leqslant 0.05$ and changes $\geqslant$2-fold (either up- or down-regulated) as cut-off values. For DNA-array analysis and representation, p values are generated using a parametric one-way ANOVA whereby the variances between groups are not assumed to be equal. V-plot graphs are color-coded

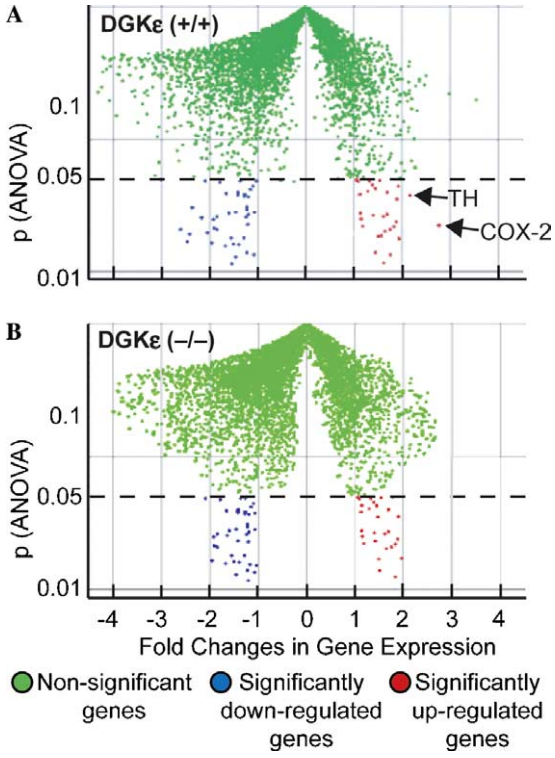

Fig. 1. Volcano plots of (A) kindled DGKε (+/+) versus control DGKε (+/+) mice and (B) kindled DGKε (−/−) versus control DGKε (−/−) mice in whole hippocampus. Arrows point to up-regulated oxygenase-encoding genes TH and COX-2; for clarity the data of non-significant genes in the region $p \leqslant 0.05$ (dashed horizontal line) and fold-changes ≤1.0 are not shown.

by significance in order to highlight up- and down-regulated genes (by convention, color-coded red and blue, respectively). Genes excluded from this stringent analysis are termed 'non-significant genes' and are by convention color-coded green. Fig. 1A shows the V-plot of kindling vs control in DGKε (+/+) animals and Fig. 1B shows the V-plot of kindling vs control in DGKε (−/−) animals.

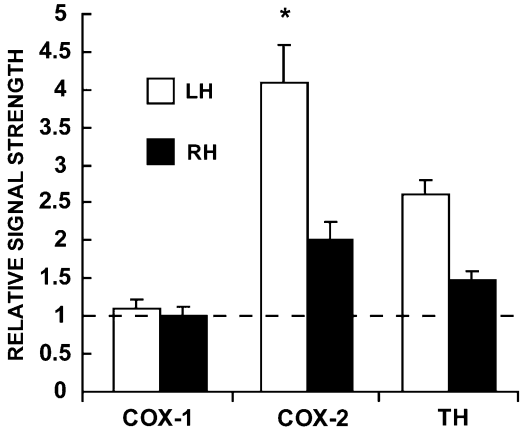

Fig. 2. Relative signal strength refers to fold-changes in the expression of COX-1, COX-2, and TH RNA in control and kindled DGKε (+/+) mice compared against βTUBIII (control) levels in the same sample. LH, left hippocampus; RH, right hippocampus; *$p < 0.05$.

Using the experimental cut-off of $p \leqslant 0.05$ and changes $\geqslant$2-fold (either up- or down-regulated), only four genes achieved these cut-off values in kindling vs control in DGKε (+/+) animals; and two of these encoded the oxygenases COX-2 and TH (Fig. 2). Interestingly, animals kindled with electrode units in the right dorsal hippocampus showed higher levels of both COX-2 and TH in the ipsilateral (left) hippocampus, when compared against βTUBIII RNA levels in the same sample (Fig. 2). No changes in the levels of the 'housekeeping' genes βTUBIII or COX-1 RNA were noted in either the left or right hippocampus after kindling. After Western immunoblot analysis of the ipsilateral (left) hippocampus, up-regulation in the RNA for COX-2 and TH was indeed reflected in higher COX-2 and TH protein levels; however, no corresponding increases were noted for βTUBIII, COX-1, or COX-3 protein (Fig. 3).

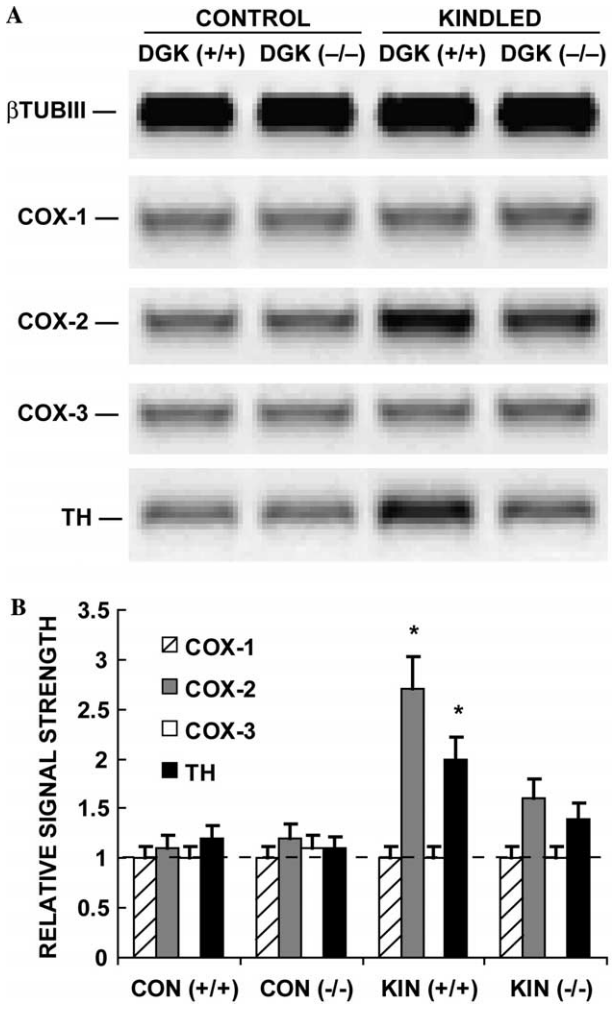

Fig. 3. (A) Western analysis of COX-1, COX-2, COX-3, and TH protein in control and kindled, DGKε (+/+) and DGKε (−/−) mice. (B) Graphed representation of data. COX-1, COX-2, COX-3, and TH each migrated as one single major band (as shown). All fold-changes were compared to that of COX-1 proteins levels in DGKε (+/+) animals to which was assigned the arbitrary value of 1.0 (dashed horizontal line). $N = 3$; $^*p < 0.05$.

Discussion

In this study, using DNA arrays, we used a global gene-expression screening strategy to search for brain-enriched genes that showed significantly altered RNA abundance levels in kindled DGKε (+/+) and DGKε (−/−) mice vs unkindled controls. From this initial gene screening, we further identified specific oxygenases that were significantly up- or down-regulated. In agreement with our previous studies using the Murine Genome U74Av2 GeneChip array (Affymetrix; 12,000 gene targets; unpublished observations), a surprisingly small number of genes reached a significance of twofold or greater (either up- or down-regulated) with an ANOVA (p) $\leqslant 0.05$ under the experimental conditions described. Among the most significantly up-regulated oxygenase genes in DGKε-knockout (−/−) mice included those encoding the inducible prostaglandin synthase COX-2 and tyrosine hydroxylase (TH; also known as tyrosine 3-monooxygenase), the first enzyme in the pathway of catecholamine synthesis. That DGKε (−/−) mice show higher resistance to electroconvulsive shock with shorter tonic seizures and faster recovery than do DGKε (+/+) mice suggests that the major catalytic end-products of COX-2 and TH, prostaglandins and catecholamines, respectively, contribute significantly toward seizure development, activity, and/or propagation.

Cyclooxygenase isozymes and epilepsy

Cyclooxygenase (COX) enzymes are critical regulators of the inflammatory and immune response, fever, and pain, and are also ubiquitous autocrine/paracrine modulators of cellular responses that are likely to play roles in mitogenesis and apoptosis [4–7,11]. In the brain, the COX superfamily of prostaglandin synthase genes encodes a constitutively expressed COX-1 active in the endoplasmic reticulum, an inducible, highly regulated, immediate-early gene COX-2, and a COX-3 isoform whose RNA is derived through the retention of a highly structured, G + C-rich intron 1 of the COX-1 gene [4,5,13,14]. COX-3 gene expression is enriched in the cerebral microvasculature [4,13]. Recent findings demonstrate that COX enzymes are of primary importance in the brain's unique inflammatory response, and these play determinant roles in the proliferation of oxidative stress, neural degeneration, and brain cell apoptosis [11–14]. Moreover, COX isozymes are the targets of non-steroidal anti-inflammatory drugs (NSAIDs) and may be of therapeutic value after neural injury [4,5,13]. Neuronal COX-2 transiently increases in a synaptic activity-dependent fashion and is also up-regulated during kainic acid-induced hippocampal cell damage [3,5]. Up-regulated brain COX-2 and increased abundance of the major COX-2-generated PGE2 are associated with the recurrence of hippocampal seizures [15], in part by selectively altering fundamental membrane/synaptic properties in the hippocampus that dynamically regulate membrane excitability [6,15]. Selective COX-2 inhibitors have been shown to

significantly reduce postsynaptic membrane excitability, back-propagating dendritic action potential-associated Ca^{2+} influx, and long-term potentiation induction in hippocampal dentate granule neurons [7], suggesting a potential role for COX-2-selective NSAIDs in epilepsy management.

Tyrosine hydroxylase and epilepsy

Although the basal ganglia appear unable to directly initiate or propagate seizures, abundant data suggest that kindling modifies the basal excitability of this modifier system [16–18]. Alam and Starr [19] were the first to report dopaminergic modulation of induced seizures in the rat and the role of the hippocampal dopamine D1 receptors in induced seizure mediation. Seizures are known to modify brain dopamine function in both humans and rats, and hippocampal dopamine appears to lower seizure threshold via activation of the dopamine D1 receptor [19,20]. Up-regulated tyrosine hydroxylase (TH) (tyrosine 3-monooxygenase), the rate-limiting enzyme for the conversion of tyrosine to the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) in neural tissues, suggests that up-regulation of TH gene expression in kindled hippocampus may represent a homeostatic adaptation to compensate for the seizure threshold-lowering effects of the kindling process [20].

Summary

The observation that the vast majority of all brain hippocampal genes, after kindling stimulation, show relatively small changes in gene expression suggests that basal levels of global gene expression in mouse brain may be reset after electrical stimulation, and this may contribute to downstream signaling events in the kindling process. Kindling epileptogenesis appears to be accompanied by the specific up-regulation of COX-2 and TH gene expression, and that these changes were more prominent in DGKε (+/+) mice when compared to DGKε (−/−) mice suggests the requirement for DGKε gene expression and/or signaling in COX-2 and TH gene regulation. Levels of the catalytic products of COX-2 and TH, namely PG and dopamine, are relatively abundant in kindled mouse brain hippocampus (Fig. 3) and are known to be altered during kainate-induced seizures in rodents and in other animal models of epilepsy [15,21,22]. These data provide additional mechanistic insight into the basic genetic mechanisms of kindling epileptogenesis and reveal novel pathways for anti-epileptogenic drug discovery. For example, there is an emerging role of COX-2 and COX-2-generated PG in the regulation of membrane excitability in hippocampal perforant path-dentate gyrus synapses [3,5–7,21]. Coupled to the observed reduction of postsynaptic membrane excitability and back-propagating dendritic action potential-associated Ca^{2+} influx in hippocampal neurons after the use of selective COX-2 inhibitors, this suggests that COX-2-specific antagonists (such as NSAIDs) may have some therapeutic value for use as anti-epileptogenic drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.08.109.

References

[1] E.B. Rodriguez de Turco, W. Tang, M.K. Topham, F. Sakane, V.L. Marcheselli, C. Chen, A. Taketomi, S.M. Prescott, N.G. Bazan, Diacylglycerol kinase epsilon regulates seizure susceptibility and long-term potentiation through arachidonoyl-inositol lipid signaling, Proc. Natl. Acad. Sci. USA 98 (2001) 4740–4745.
[2] W.J. van Blitterswijk, B. Houssa, Diacylglycerol kinases in signal transduction, Chem. Phys. Lipids 98 (1999) 95–108.
[3] B. Tu, N.G. Bazan, Hippocampal kindling epileptogenesis upregulates neuronal cyclooxygenase-2 expression in neocortex, Exp. Neurol. 179 (2003) 167–175.
[4] B. Kis, J.A. Snipes, D.W. Busija, Acetaminophen and the COX-3 puzzle: sorting out facts, fictions and uncertainties, J. Pharmacol. Exp. Ther. (2005) [Epub ahead of print].
[5] D.L. Simmons, R.M. Botting, T. Hla, Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition, Pharmacol. Rev. 56 (2004) 387–437.
[6] C. Chen, N.G. Bazan, Endogenous prostaglandin E2 regulates membrane excitability and synaptic transmission in hippocampal CA1 pyramidal neurons, J. Neurophysiol. 93 (2005) 929–941.
[7] C. Chen, J.C. Magee, N.G. Bazan, Cyclooxygenase-2 regulates prostaglandin E2 signaling in hippocampal long-term synaptic plasticity, J. Neurophysiol. 87 (2002) 2851–2857.
[8] C. Deransart, G.B. Landwehrmeyer, T.J. Feuerstein, C.H. Lucking, Up-regulation of D3 dopaminergic receptor mRNA in the core of the nucleus accumbens accompanies the development of seizures in a genetic model of absence-epilepsy in the rat, Brain Res. Mol. Brain Res. 94 (2001) 166–177.
[9] P. DE Lonlay, P.M.C. Nassogne, A.H. van Gennip, A.C. van Cruchten, T. Billatte de Villemeur, M. Cretz, C. Stoll, J.M. Launay, G.C. Steenberger-Spante, L.P. van den Heuvel, R.A. Wevers, J.M. Saudubray, N.G. Abeling, Tyrosine hydroxylase deficiency unresponsive to L-dopa treatment with unusual clinical and biochemical presentation, J. Inherit. Metab. Dis. 23 (2000) 819–825.
[10] J.R. Ryu, C.Y. Shin, K.H. Park, G.S. Jeon, H. Kim, W. Kim, J.W. Dailey, P.C. Jobe, S.S. Cho, K.H. Ko, Effect of repeated seizure experiences on tyrosine hydroxylase immunoreactivities in the brain of genetically epilepsy-prone rats, Brain Res. Bull. 53 (2000) 777–782.
[11] N.G. Bazan, W.J. Lukiw, Cyclooxygenase-2 and presenilin-1 gene expression induced by interleukin-1beta and amyloid beta 42 peptide is potentiated by hypoxia in primary human neural cells, J. Biol. Chem. 277 (2002) 30359–30367.
[12] V. Colangelo, J. Schurr, M.J. Ball, R.P. Pelaez, N.G. Bazan, W.J. Lukiw, Gene expression profiling of 12633 genes in Alzheimer

hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling, J. Neurosci. Res. 70 (2002) 462–473.
[13] J.G. Cui, H. Kuroda, N.V. Chandrasekharan, R.P. Pelaez, D.L. Simmons, N.G. Bazan, W.J. Lukiw, Cyclooxygenase-3 gene expression in Alzheimer hippocampus and in stressed human neural cells, Neurochem. Res. 29 (2004) 1731–1737.
[14] N.G. Bazan, V. Colangelo, W.J. Lukiw, Prostaglandins and other lipid mediators in Alzheimer's disease, Prostaglandins Other Lipid Mediat. 68–69 (2002) 197–210.
[15] T. Takemiya, K. Suzuki, H. Sugiura, S. Yasuda, K. Yamagata, Y. Kawakami, E. Maru, Inducible brain COX-2 facilitates the recurrence of hippocampal seizures in mouse rapid kindling, Prostaglandins Other Lipid Mediat. 71 (2003) 205–216.
[16] C. Deransart, A. Depaulis, The control of seizures by the basal ganglia? A review of experimental data, Epileptic Disord. 3 (2002) S61–S72.
[17] M. Gernert, M. Fedrowitz, P. Wlaz, W. Loscher, Subregional changes in discharge rate, pattern, and drug sensitivity of putative GABAergic nigral neurons in the kindling model of epilepsy, Eur. J. Neurosci. 20 (2004) 2377–2386.
[18] C. Freichel, U. Ebert, H. Potschka, W. Loscher, Amygdala-kindling does not induce a persistent loss of GABA neurons in the substantia nigra pars reticulata of rats, Brain Res. 1025 (2004) 203–209.
[19] A.M. Alam, M.S. Starr, Dopaminergic modulation of pilocarpine-induced motor seizures in the rat: the role of hippocampal dopamine D1 receptors, Eur. J. Pharmacol. 222 (1992) 227–232.
[20] F.S. Giorgi, M. Ferrucci, G. Lazzeri, C. Pizzanelli, P. Lenzi, M.G. Alessandrl, L. Murri, F. Fornai, A damage to locus coeruleus neurons converts sporadic seizures into self-sustaining limbic status epilepticus, Eur. J. Neurosci. 17 (2003) 2593–2601.
[21] M.G. Naffah-Mazzacoratti, M.I. Bellissimo, E.A. Cavalheiro, Profile of prostaglandin levels in the rat hippocampus in pilocarpine model of epilepsy, Neurochem. Int. 27 (1999) 461–466.
[22] R.M. Kaminski, T.S. Shippenberg, J.M. Witkin, B.A. Rocha, Genetic deletion of the norepinephrine transporter decreases vulnerability to seizures, Neurosci. Lett. 382 (2005) 51–55.