Brain MAPKs Levels are Differentially Associated with Seizures Threshold and Severity Progression in Pentylenetetrazole-Kindled Mice

Juliana Ben,¹ Filipe Marques Gonçalves,² Paulo Alexandre Oliveira,³ Tanara Vieira Peres,¹ Alexandre Hohl,^{4,5} Rodrigo Bainy Leal,^{1,2,5} Esper Abrão Cavaleiro,⁶ Rui Daniel Schroder Prediger^{1,3,5} & Roger Walz^{1,4,5}

1 Programa de Pós-Graduação em Neurociências, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, Brazil

2 Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, Brazil

3 Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, Brazil

4 Departamento de Clínica Médica, Centro de Ciências da Saúde, Hospital Universitário (HU), Universidade Federal de Santa Catarina, Florianópolis, Brazil

5 Centro de Neurociências Aplicadas (CeNAp), Hospital Universitário (HU), Florianópolis, Brazil

6 Disciplina de Neurologia Experimental, Escola Paulista de Medicina/Universidade Federal de São Paulo (EPM/UNIFESP), São Paulo, Brazil

Correspondence

R. Walz, M.D., Ph.D., Departamento de Clínica Médica, Hospital Universitário, 3 andar, UFSC, Campus Universitário, Trindade Florianópolis, Brazil.

Tel.: +55-48-3721-9149;

Fax: +55-48-3721-9014;

E-mail: rogerwalz@hotmail.com

Received 2 May 2013; revision 28 May 2013;

accepted 29 May 2013

doi: 10.1111/cns.12147

Epilepsies are characterized by unprovoked recurrent epileptic seizures showing a prevalence of 5–10 cases per 1000 inhabitants in developed countries, which may be even higher in developing countries [1]. Despite their antiseizure effects, antiepileptic drugs (AEDs) had no clinically proved effects to prevent or reverse the epileptogenesis [2]. This indicates that AEDs mechanisms affecting seizure threshold may differ at least in part from those involved with epileptogenesis.

The kindling is a process of progressive and persistent intensification of seizure susceptibility during repeated chemical or electrical subthreshold stimulation, resulting in generalized seizures [3,4]. Although it is not proven that kindling process can occur in humans, kindling models have been extensively used for understanding the basic mechanisms of epileptogenesis as well pharmacological screening for new AEDs. The mitogen-activated protein kinases (MAPKs) are a group of serine–threonine kinases that amplify and integrate signals originating from a variety of extracellular stimuli. They regulate several processes in the CNS including cell differentiation, survival, cell death, and synaptic plasticity. They consist of three main kinases subfamilies, the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the c-Jun-N-terminal kinases 1–3 (JNK1/2/3), and the p38 kinases α – δ (p38^{MAPK} α /b/c/d) [5–8]. Changes of these cellular cascades systems have been associated with the time course-related epileptogenesis process in rodents [6,9,10].

The pentylenetetrazol, a blocker of the chloride ionophore channel of the GABA_A receptor, is worldwide used in chemical kindling protocols in rodents [3,4]. Here, we investigated whether the total and phosphorylated levels of ERK1, ERK2, JNK1, JNK2, p38 in the cortex and hippocampus of PTZ-kindled mice were differentially associated with (1) kindling progression estimated by the number of days with convulsive seizures and (2) seizure latency determined by the time course (in seconds) between the PTZ injection and the convulsive seizure start in the last stimulation day.

All procedures were carried out between 9–12 a.m. and according to UFSC Ethics Committee. Adult male Swiss mice ($n = 24$) received a subconvulsive dose of PTZ (35 mg/kg, i.p.) on alternate days, and the increasing of epileptic seizures intensity (Racine stage 1–5) with each administration was monitored behaviorally during 1 h as previously described [3,4]. Control animals ($n = 4$) received eight saline injections also on alternate days. After the 8 days of PTZ stimulation, two animals died, three had only one convulsive seizure and four were resistant to kindling. The remained 15 kindled animals and controls were euthanized by decapitation 48 h after the last injection and their cortex and hippocampus were removed for the neurochemical analysis. This time course between the last injection and the biologic samples collection was based on our previous observation (data not shown) that MAPKs parameters already return to basal levels after

Table 1 Variables associated with the number of days with seizure in PTZ-kindled mice

Predictive variables	R	R ²	B coefficient	"P" level
Latency for the last seizure onset	0.45	0.20	−0.004	0.09
Latency for the first seizure onset	0.09	0.009	0.000	0.74
MAPKs levels in the cortex				
Total ERK1	0.05	0.003	−0.02	0.85
Phospho-ERK1	0.32	0.10	−0.02	0.24
Total ERK2	0.45	0.20	−0.18	0.09
Phospho-ERK2	0.28	0.08	−0.02	0.31
Total JNK1	0.21	0.04	−0.08	0.45
Phospho-JNK1	0.03	0.001	0.004	0.91
Total JNK2	0.52	0.07	−0.15	0.05
Phospho-JNK2	0.08	0.007	−0.01	0.76
Total p38	0.10	0.01	0.007	0.71
Phospho-p38	0.33	0.11	−0.01	0.22
MAPKs levels in the hippocampus				
Total ERK1	0.06	0.004	0.007	0.82
Phospho-ERK1	0.56	0.31	−0.01	0.03
Total ERK2	0.13	0.02	0.009	0.63
Phospho-ERK2	0.32	0.10	−0.008	0.24
Total JNK1	0.35	0.12	0.03	0.20
Phospho-JNK1	0.36	0.13	−0.02	0.19
Total JNK2	0.35	0.12	0.02	0.20
Phospho-JNK2	0.49	0.25	−0.04	0.06
Total p38	0.50	0.25	−0.02	0.06
Phospho-p38	0.23	0.05	−0.006	0.41

a single PTZ-convulsion, excluding any residual effects of PTZ or the epileptic seizure on the neurochemical parameters.

The total and phosphorylated MAPKs were determined by Western blotting as previously described [6]. The whole cellular content of neocortex and hippocampus homogenized in 400 μ l of Tris 50 mM pH 7.0, EDTA 1 mM, NaF 100 mM, PMSF 0.1 mM, Na₃VO₄

Table 2 Variables associated with the latency for the seizure onset of the last convulsive seizure in PTZ-kindled mice

Predictive variables	R	R ²	B coefficient	"P" level
Number of days with seizure	0.45	0.20	−22.3	0.01
Latency for the first seizure onset	0.63	0.39	0.46	0.01
MAPKs levels in the cortex				
Total ERK1	0.40	0.16	−14.6	0.14
Phospho-ERK1	0.26	0.07	1.77	0.35
Total ERK2	0.41	0.17	18.9	0.13
Phospho-ERK2	0.10	0.01	0.93	0.71
Total JNK1	0.08	0.006	−3.27	0.78
Phospho-JNK1	0.05	0.003	0.63	0.86
Total JNK2	0.21	0.04	6.79	0.46
Phospho-JNK2	0.09	0.008	1.31	0.75
Total p38	0.62	0.40	−4.52	0.01
Phospho-p38	0.71	0.50	2.47	0.003
MAPKs levels in the hippocampus				
Total ERK1	0.36	0.13	−4.15	0.19
Phospho-ERK1	0.23	0.05	0.69	0.41
Total ERK2	0.17	0.03	1.35	0.54
Phospho-ERK2	0.06	0.004	−0.17	0.83
Total JNK1	0.12	0.01	1.06	0.66
Phospho-JNK1	0.02	0.000	0.12	0.95
Total JNK2	0.14	0.02	−1.38	0.61
Phospho-JNK2	0.35	0.12	1.95	0.20
Total p38	0.18	0.03	1.20	0.51
Phospho-p38	0.18	0.03	0.56	0.52

2 mM, Triton X-100 1%, glycerol 10%, Sigma protease inhibitor cocktail and then incubated for 10 min in ice. Lysates were centrifuged (10,000 g for 10 min, at 4°C) to eliminate cellular debris. All membranes were incubated with mouse anti- β -actin antibody to control the proteins amounts loaded on the gel. The MAPKs quantifications by the Scion Image software were expressed as optic density (OD) percentage of controls. The phosphorylation

Table 3 Predictive model showing the variables independently associated with number of days with seizure in PTZ-kindled mice

Predictive variables	B coefficient (CI 95%)	"P" level
Constant	−6.27 (−15.58 to −3.04)	0.16
MAPKs levels in the hippocampus		
Phospho-ERK1	−0.02 (−0.04 to −0.005)	0.003
Phospho-JNK2	−0.08 (−0.12 to −0.03)	0.002
Total JNK1	0.08 (0.02 to 0.13)	0.009
Phospho-JNK1	0.13 (0.06 to 0.21)	0.001

Model summary: $R = 0.87$, $R^2 = 0.75$, $F = 7.58$, $P = 0.004$

Representative figures of MAPKs immunoblottings

Table 4 Variables independently associated with the latency for seizure onset of the last convulsive seizure in PTZ-kindled mice

Predictive variables	B coefficient (CI 95%)	"P" level
Constant	−34.9 (−192.4 to 122.4)	0.64
Latency for the first seizure onset	0.30 (0.001 to 0.61)	0.04
MAPK levels in the Cortex Phospho-p38	1.90 (0.44 to 3.35)	0.01

Model summary: $R = 0.80$, $R^2 = 0.64$, $F = 10.4$, $P = 0.002$

Representative figures of MAPKs
immunoblottings

levels of MAPKs were determined as a ratio of OD of phosphorylated band/OD of total band [6].

All data analyzed showed a normal distribution (one-sample Kolmogorov–Smirnov test, $P > 0.25$). The univariate analysis was done by linear regressions to identify the neurochemical variables associated with kindling progression and the latency for the last seizure onset at a "P" level ≤ 0.20 (see bold values in the Tables 1 and 2). Thereafter, these variables were included in the multiple linear regressions analysis to identify the MAPKs independently associated with kindling progression and latency for the last seizure occurrence. Because of the biologic plausibility, we also included in the multivariate analysis the variable latency for the seizure onset in the first day with convulsive seizure (Racine 4 or 5). To minimize spurious association due to the multiple comparisons, only "P" levels lower than 0.01 were considered statistically significant in the final model of logistic regression. Analyses were done by SPSS 17.0 (Chicago, IL, USA).

The final multiple linear regression model including the total JNK2 in the cortex and phospho-ERK1, phospho-JNK2, total JNK1 and phospho-JNK1 in the hippocampus showed a very strong association ($R = 0.88$) with the kindling progression estimated by the number of days with Racine stage 4 or 5 in kindled mice (Table 3). These variables explained 66% (adjusted $R^2 = 0.66$) of the variance in the number of days with seizure ($F = 6.92$, $P = 0.005$).

The final multiple linear regression model including the cortex phospho-p38 level and the latency for the seizure occurrence after PTZ injection in the first day with Racine stage 5 seizure showed a very strong association ($R = 0.80$) to the latency for seizure onset after the last PTZ injection (Table 4). These variables together explained 65% (adjusted $R^2 = 0.65$) of the variance of latency for

seizure onset (Racine stage 5) in the last stimulation day of PTZ-kindled mice ($F = 11.9$, $P = 0.001$).

The experimental design applied here does not confirm a cause–effect relationship among the brain MAPKs levels and kindling development. However, our findings may indicate that brain MAPKs levels variations are predictors' biomarkers of the PTZ-kindling development parameters in mice. The independent predictive power of these biomarkers is variable according to the analyzed parameters (number of days with seizures or latency for the last seizure onset) raising the possibility that mechanisms involved with epileptogenesis and seizure threshold may differ in the PTZ-kindling. The mechanisms may involve differential changes in the total or activated MAPKs content according to the cortical and subcortical brain region. These findings may be, at least in part, related to the lack of clinically relevant effects of antiseizures drugs against an installed epileptogenesis.

Acknowledgments

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), IBN.Net/CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Projeto CAPES/PROCAD, Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC-PRONEX, NENASC Project) and Instituto Nacional de C&T de Neurociência Translacional.

Conflict of Interest

The authors declare no conflict of interest.

References

- Bartolini E, Bell GS, Sander JW. Multicultural challenges in epilepsy. *Epilepsy Behav* 2011;20:428–434.
- Galanopoulou AS, Buckmaster PS, Staley KJ, et al. Identification of new epilepsy treatments: issues in preclinical methodology. *Epilepsia* 2012;53:571–582.
- Bonan CD, Amaral OB, Rockenbach IC, et al. Altered ATP hydrolysis induced by pentylenetetrazol kindling in rat brain synaptosomes. *Neurochem Res* 2000;25:775–779.
- Walz R, Amaral OB, Rockenbach IC, et al. Increased sensitivity to seizures in mice lacking cellular prion protein. *Epilepsia* 1999;40:1679–1682.
- Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298:1911–1912.
- Lopes MW, Soares FM, de Mello N, et al. Time-dependent modulation of mitogen activated protein kinases and AKT in rat hippocampus and cortex in the

- pilocarpine model of epilepsy. *Neurochem Res* 2012;**37**:1868–1878.
7. Martin KC, Michael D, Rose JC, et al. MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in *Aplysia*. *Neuron* 1997;**18**:899–912.
8. Walz R, Lenz G, Roesler R, et al. Time-dependent enhancement of inhibitory avoidance retention and MAPK activation by post-training infusion of nerve growth factor into CA1 region of hippocampus of adult rats. *Eur J Neurosci* 2000;**12**:2185–2189.
9. Garrido YC, Sanabria ER, Funke MG, Cavalheiro EA, Naffah-Mazzacoratti MG. Mitogen-activated protein kinase is increased in the limbic structures of the rat brain during the early stages of status epilepticus. *Brain Res Bull* 1998;**47**:223–229.
10. Walz R, Castro RM, Velasco TR, et al. Cellular prion protein: implications in seizures and epilepsy. *Cell Mol Neurobiol* 2002;**22**:249–257.