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MYOCYTE-SPECIFIC ENHANCER BINDING FACTOR 2C (MEF2C) EXPRESSION IN THE DENTATE GYRUS DURING DEVELOPMENT AND AFTER PILOCARPINE-INDUCED *STATUS EPILEPTICUS*

A preliminary report

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Abstract – Objective: As axon outgrowth and dentate granule cell neurogenesis are hallmarks of hippocampal development and are also the two morphologic changes in the structure of the dentate gyrus after *status epilepticus* (SE), we hypothesized that molecules involved in normal development may also play a role during epileptogenesis. **Method:** Using *in situ* hybridization, we have characterized mRNA expression of myocyte-specific enhancer binding factor 2C (MEF2C) in the dentate gyrus during development (P0, P3, P7, P14 and P28) and at multiple time points following pilocarpine-induced SE (3, 7, 14, 28 days after SE). **Results:** It was demonstrated that MEF2C is up-regulated during development (P0, P3, P7, P14 and P28) and in the adult rat dentate gyrus following SE (3, 7, 14, 28 days after SE). **Conclusions:** The molecules controlling cell-fate decisions in the developing dentate gyrus are also operative during epileptogenesis.

KEY WORDS: rat, hippocampus, epilepsy, *status epilepticus*, MEF2C.

Realce miócito específico ligado a expressão do fator 2C (MEF2C) no giro denteado durante o desenvolvimento e após uso de pilocarpina induzindo *status epilepticus:* estudo preliminar

Resumo – Objetivo: Como o crescimento axonal e a neurogênese do giro denteado são características intrínsecas do hipocampo durante o processo de desenvolvimento, e também são duas alterações morfológicas na estrutura do giro denteado após o *status epilepticus* (SE), nós hipotetizamos que as moléculas envolvidas no processo normal do desenvolvimento hipocampal também podem participar do processo de epileptogênese. **Método:** Utilizando hibridização *in situ*, caracterizamos a expressão do RNAm do fator de transcrição *myocyte-specific enhancer binding factor 2C* (MEF2C) no giro denteado durante o desenvolvimento (P0, P3, P7, P14 e P28) e em diferentes períodos após o SE (3, 7, 14, 28 dias após SE). **Resultados:** Foi demonstrado um aumento da expressão de MEF2C no giro denteado durante o desenvolvimento e no giro denteado de animais adultos após o SE. **Conclusão:** As moléculas que controlam o destino celular durante o processo de desenvolvimento também estão operativas durante o processo de epileptogênese.

PALAVRAS-CHAVE: rato, hipocampo, epilepsia, *status epilepticus*, MEF2C.

Epilepsy is the commonest serious neurological condition. Temporal lobe epilepsy (TLE), the most common epilepsy in adults, is generally intractable and is suspected to be the result of recurrent excitation or inhibition circuitry[1]. TLE is defined by recurring partial complex seizures arising from limbic structures of the temporal lobes, including the hippocampus, dentate gyrus, amygdala and associated temporal neocortex[1]. The study of mechanisms of the epilepsies requires employment of animal models. In these lines, the pilocarpine model is the most useful TLE model that reflects the human condition[2]. In brief, the systemic administration of the cholinergic agonist pilocarpine to rats promotes sequential behavioural and electrographic changes that can be divided into three distinct

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periods: a) an acute period that builds progressively into limbic *status epilepticus* (SE) and that lasts 24h; b) a silent period with progressive normalization of EEG and behaviour which varies from 4 to 44 days; and c) a chronic period with spontaneous recurrent seizures[2]. From a morphological point of view, seizure-induced injury leads to various forms of plasticity in the adult rodent dentate gyrus[3]. While these forms of neuroplasticity during epileptogenesis have been well documented, the molecular mechanisms underlying these alterations remain poorly understood.

During the last decade, a number of transcription factors have been shown to be involved in the cellular and molecular organization of the brain. The myocyte-specific enhancer binding factor 2C (MEF2C), a member of the MEF2 sub-family of the MADS gene family, is a transcription factor expressed at high levels in the brain and has been implicated in the development of neurons[4]. MEF2C binds with basic helix-loop-helix (bHLH) proteins[5], which in the brain are responsible for neural determination or differentiation[6]. There is more definitive evidence for interactions among MEF2 with bHLH factors. For example, multiple members of bHLH family of transcription factors have been shown to be expressed in dentate granule cells at specific developmental stages[7], supporting their involvement in regulating dentate gyrus development. Interestingly, Elliott and colleagues[8] demonstrated that the expression of bHLH molecules is altered during seizure-associated network reorganization. Specifically, they showed varied profiles of expression of bHLH family members following SE, with some increasing (Mash1, Id2), some decreasing (Hes5, Prox1), and others remaining unchanged NeuroD/BETA2, NeuroD2/NDRF, Id3, Rath2/Nex1, supporting the idea that molecules controlling cell-fate decisions in the developing dentate gyrus are also operative during seizure-induced plasticity.

As MEF2C has been implicated to play an important role in the process of neuronal differentiation, we pursued the hypothesis that MEF2C is a potential upstream regulator of bHLH expression that is modified following SE. To do so, using *in situ* hybridization, we have characterized mRNA expression of MEF2C in the dentate gyrus during development and at multiple time points following pilocarpine-induced SE.

METHOD

Animals

For adult animals (n=40), two groups of Sprague-Dawley rats (180-200g) were studied: (A) Adult male rats at different time points following pilocarpine-induced *status epilepticus* (SE). It is important to note that for each time point (3, 7, 14, 28 days after SE) we used n=8 animals, (B) Control group, adult male rats (n=8), that received saline instead pilocarpine. For postnatal ages, subjects were male Sprague-Dawley rats (n=25) with ages P0, P3, P7, P14 and P28 days and for each group we used n=5 animals. The rats were bred in our laboratories and the day of birth was considered as day 0. The pups were housed with their mother in individual cages until weaning at day 21. All the animals used in our study were housed under standard controlled conditions (7:00 AM/7:00 P.M. light/dark cycle; 20-22ºC; 45–55% humidity) with food and water *ad libitum*.

Induction of SE

All animals were treated according to protocols for animal care established by the Harvard Medical School, Boston and the National Institutes of Health, and all efforts were made to minimize animal suffering. Adult male Sprague-Dawley rats (180–200g) were given an intraperitoneal (i.p.) injection of atropine methylbromide (5 mg/kg; Sigma, St. Louis, MO, USA) followed 20 minutes later by an i.p. injection of pilocarpine hydrochloride (340 mg/kg; Sigma) to induce SE. Seizure activity was monitored behaviourally and terminated with an i.p. injection of diazepam (10 mg/kg; Elkins-Sinn, Cherry Hill, NJ, USA) after 2 hr of convulsive SE. Only rats that displayed continuous, convulsive seizure activity after pilocarpine treatment were included in these studies. Control animals received the same injections of atropine and diazepam, but received saline instead of pilocarpine.

Tissue preparation

At designated time points following SE, all animals received an anesthetic overdose of pentobarbital and transcardially perfused with 300 mL of a 4% paraformaldehyde solution in phosphate-buffered saline at pH 7.4. Brains were post-fixed *in situ* overnight at 4ºC and then removed and cryoprotected in 30% sucrose in PBS prior to freezing. To generate anatomically comparable sections for the *in situ* hybridization analysis, 20 µm frozen coronal sections were cut through the middle third of the hippocampus with a cryostat, melted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at –20ºC for subsequent *in situ* analysis of mRNA. Potential variations in mRNA expression along the septo/temporal axis of the hippocampus were not assessed in this study.

Non-radioactive *in situ* hybridization

Non-radioactive *in situ* hybridization was performed essentially as described previously[7] using a protocol obtained from Dr. David J. Anderson (California Institute of Technology). Briefly, sections were pre-treated with Proteinase K for 5 minutes prior to prehybridization for 3 hr in a solution containing yeast tRNA, Denhardt's solution, and 50% formamide. Following prehybridization, sections were incubated at their respective optimal temperatures overnight with digoxigenin-labeled probes at a final concentration of 1 µg/µl. Slides were washed the next day at high stringency and incubated with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (diluted 1:2000; Roche Molecular Biochemicals, Indianapolis, IN, USA) for 2-3 hr at room temperature. Following washes, the slides were incubat-

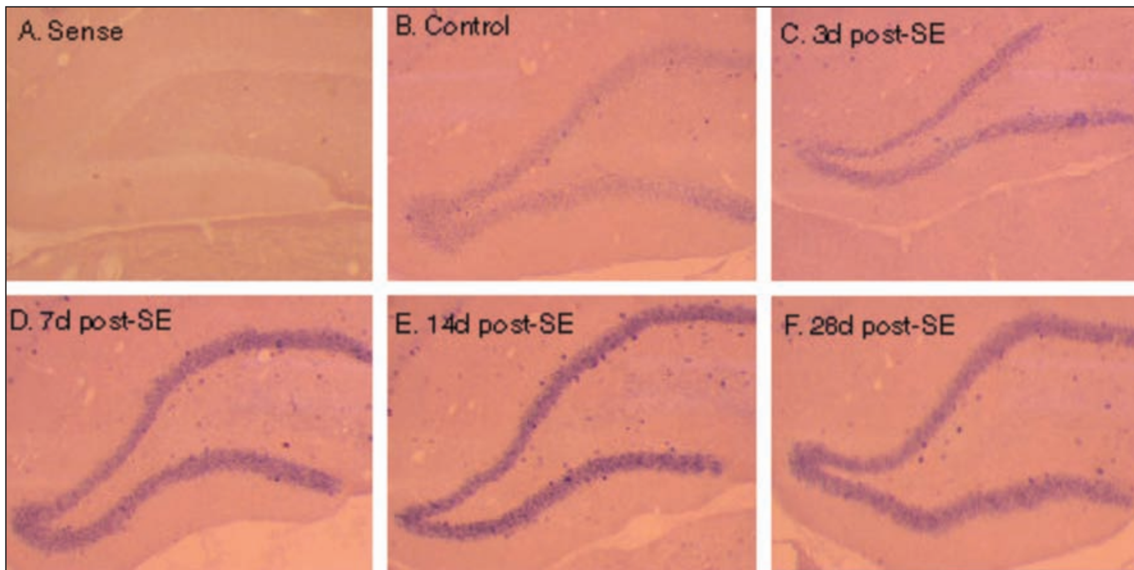

Fig 1. Expression MEF2C mRNA in dentate gyrus. (A) In situ hybridization of 14 days post-SE tissue with MEF2C sense riboprobe shows the representative lack of signal and minimal background staining. (B) In situ hybridization of control tissue with MEF2C antisense riboprobe demonstrates the expression of MEF2C troughout the dentate granule cell layer. (C) At 3 days following SE, the MEF2C expression appears to be slightly elevated as compared to the control group. The expression of MEF2C troughout the dentate granular cell layer increases acutely within 7 days (D) and 14 days (E) following SE, remaining elevated until 28 days post – SE (F).

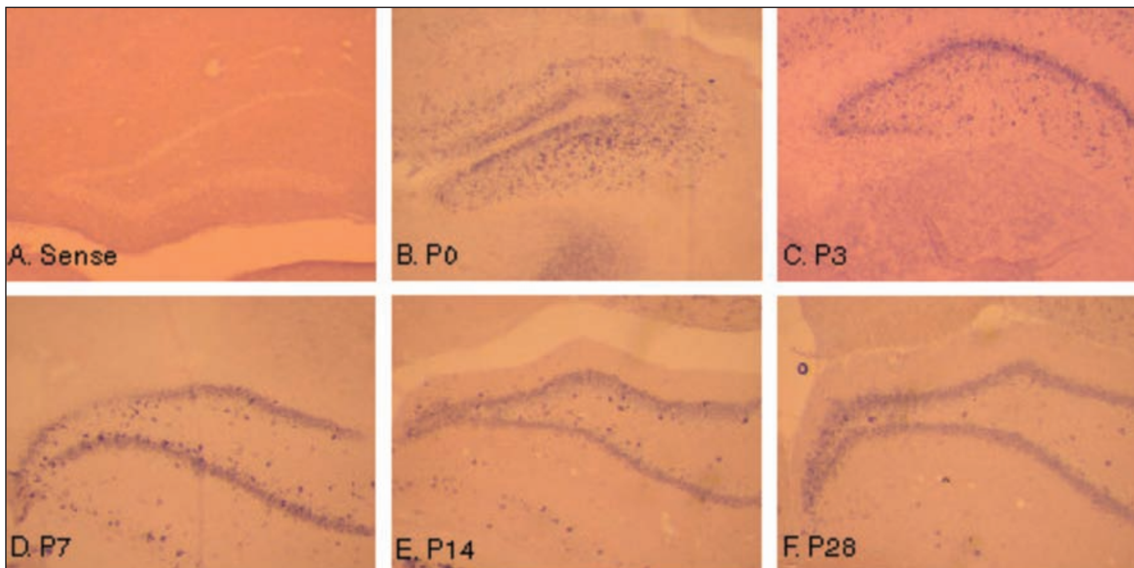

Fig 2. Expression MEF2C mRNA in dentate gyrus during development. (A) In situ hybridization of P14 tissue with MEF2C sense riboprobe shows the representative lack of signal and minimal background staining. (B) In situ hybridization of P0 tissue with MEF2C antisense riboprobe demonstrate the expression of MF2C in immature granule cells population. At P3(C) and P7 (D), MEF2C is distributed troughout the developing dentate granular cell layer. MEF2C is expressed at highest levels in granule cells in the inner of granule cell layer at P14 (E) and P28 (F).

ed in buffer containing nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (Roche Molecular Biochemicals). *In situ* data were evaluated qualitatively, based on the comparison of mRNA expression patterns in comparable hippocampal sections. These sections were taken from multiple sets of animals processed for *in situ* hybridization on different days.

Non-radioactive *in situ* probe preparation

The MEF2C probe used for this study, along with the optimal *in situ* hybridization temperatures and template sources, were described previously[9]. Probe templates generated by PCR were derived from cDNA adult rat. Antisense and sense digoxigenin-labeled RNA probes were transcribed from each template us-

ing the Genius RNA labeling kit (Roche Molecular Biochemicals) and purified with Chromaspin-100 columns packed in DEPC water (Clontech Laboratories, Palo Alto, CA, USA).

RESULTS

Pilocarpine treatment induced the following sequence of behavioral changes: akinesia, facial automatisms, and limbic seizures consisting of forelimb clonus with rearing, salivation, and masticatory jaw movements and falling. This type of behaviour built up progressively into motor limbic seizures that recurred repeatedly and rapidly and developed into *status epilepticus*. After SE, animals were unresponsive to their environment and akinetic; behavior returned toward normal over a 3 to 5-day period[2].

In situ analysis of MEF2C mRNAs at various times following SE course indicates that this molecule is expressed during epileptogenesis. *In situ* hybridization of control tissue with MEF2C antisense riboprobe demonstrates expression of MEF2C throughout the dentate granule cell layer (Fig 1B). The specificity of the probe was documented using an MEF2C sense probe, with which no signal and minimal background staining were observed (Fig 1A). In addition, at 3 days following SE the MEF2C expression in adult dentate gyrus appears to be slightly elevated as compared to the control group (Fig 1C). The expression of MEF2C mRNA throughout the dentate granule cell layer increased acutely within 7 days (Figure 1D) and 14 days (Fig 1E) following SE, and remained elevated until 28 days post-SE (Fig 1F).

In situ analysis of MEF2C during development shows that MEF2C is expressed in a specific pattern in the dentate gyrus (Fig 2A). *In situ* hybridization of P0 tissue with MEF2C antisense riboprobe demonstrates expression of MEF2C in immature granule cells populations (Fig 2B). At P3 and P7 (Figs 2C and 2D respectively), MEF2C is distributed throughout the developing dentate granule cell layer. MEF2C is expressed at highest levels in the granule cell layer at P14 (Fig 2E) and P28 (Fig 2F), when the entire inner half of the granule cell layer has only recently been generated.

DISCUSSION

It has been known that MEF2C is expressed in a dynamic pattern during development and in the mature mouse CNS, suggesting that the molecule may play an important role at different stages of neuronal differentiation and/or maturation[4,10]. Here it was demonstrated that MEF2C is up-regulated during development and in the adult rat dentate gyrus following SE, suggesting that MEF2C may also be involved in seizure-induced plasticity. Interestingly, the results in this report are not in agreement with the recent paper of Yoon and colleagues[11]. In their study, the authors have shown that MEF2C phosphorylation was reduced immediately after electroconvulsive shock. The discrepancies in these MEF2C activation patterns may reflect different epilepsy models and time points analyzed.

During development, four MEF2 genes (A, B, C, D) are expressed in spatially and temporally specific patterns during brain development, suggesting that, similar to its expression pattern in striated muscle, MEF2 gene expression in the CNS occurs in neurons exiting the cell cycle and entering differentiation[10]. In addition, human studies using immunocytochemical localization of MEF2C in hippocampal surgical specimens revealed immunoreactivity in nuclei of dentate granule cells and the hilus of dentate gyrus, supporting the concept of a role for MEF2C in postmitotic neuronal differentiation[4]. Given that, the results of this study also showed that MEF2C is expressed in dentate granule cells during specific developmental stages, it seems quite possible that MEF2C is involved is in regulating dentate gyrus development.

The persistent cell birth in the rodent dentate gyrus during postnatal and adult life is well established[12]. Interestingly, neurogenesis and mossy fiber growth are important processes that occur during both development and epileptogenesis of dentate gyrus, supporting the idea that molecules guiding neurogenesis and axon outgrowth during development overlap with those expressed during epileptogenesis[8]. Numerous examples support this hypothesis, but one of the more important of them is the differential expression of bHLH molecules during development[7] and following SE[8]. Interestingly, MEF2C binds as homodimers and heterodimers with bHLH proteins[5], supporting the idea that interactions between MEF2 factors and bHLH proteins (e.g., Mash1) are required for activation of tissue-specific transcription in neurogenic cell lineages[13]. Based on this, we believe that the increase of MEF2C expression after 3, 7, 14 and 28 days after pilocarpine-induced-SE may also be involved with dentate gyrus plasticity.

Human temporal lobe epilepsy and rodent models of limbic epilepsy are frequently associated with a marked loss of hippocampal and dentate gyrus neurons. Seizure-induced cell loss has been attributed to a mechanism of excitotoxicity-induced necrosis[14]. However, it has been shown that apoptosis contributes to the degeneration following *status epilepticus*[15] and alterations in the expression of caspases suggest an involvement of these factors in mediating apoptosis following seizures. Moreover, Ekdahl and co-workers[16] have shown that caspases modulate seizure-induced neurogenesis, possibly through the regulation of apoptosis of new neurons, because this action can be suppressed by caspase inhibitors. Along these lines, MEF2C has also been implicated in mediating or modulating apoptosis responses. Okamoto and

colleagues[17] recently described antiapoptotic functions for MEF2C. However, in mature neurons exposed to excitotoxic injury or others forms of stress, an apoptotic effect[18] mediated by caspases were demonstrated[19]. With these results, it is possible to support the hypothesis that MEF2C is involved with the mechanism of apoptotic degeneration following pilocarpine induced-SE.

Finally, the present results support previous evidence that MEF2C is expressed in areas of ongoing neural plasticity and raise, for the first time, the possibility of a potential role for MEF2C molecules during epileptogenesis. Nevertheless, there are some limitations to this study. Firstly, the present data is only from *in situ* hybridization. A description of quantification of MEF2C mRNA expression in the dentate gyrus should be carried out. Secondly, to confirm the roles of MEF2C in epileptogenesis, an imunohistochemistry study should be realized to investigate the possible changes of MEF2C protein expression in the dentate gyrus. These future studies, which are in evaluation in our laboratory, are needed to gain a better understanding of these and other possible mechanisms of MEF2C transcription factor during epileptogenesis.

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REFERENCES

1. Sutula TP. Epilepsy: a reappraisal from the perspective of neural plasticity. Int Rev Neurobiol 2001;45:355-386.
2. Cavalheiro EA. The pilocarpine model of epilepsy. Ital J Neurol Sci 1995; 16:33-37.
3. Lowenstein DH. Structural reorganization of hippocampal networks caused by seizure activity. Int Rev Neurobiol 2001;45:209-236.
4. Leifer D, Li Y, Wehr K. MEF2C expression in fetal mouse brain development. J Mol Neurosci 1997;8:1-13.
5. Molkentin J, Olson EN. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci 1996;93:9366-9373.
6. Schwab MH, Bartholomae A, Heimrich B, et al. Neuronal basic helix-loop-helix proteins (NEX and BETA2/Neuro D) regulate terminal granule cell differentiation in the hippocampus. J Neurosci 2000;20: 3714-3724.
7. Pleasure SJ, Collins AE, Lowenstein DH. Unique expression patterns of cell fate molecules delineate sequential stages of dentate gyrus development. J Neurosci 2000;20:6095-6105.
8. Elliott RC, Khademi S, Pleasure SJ, Parent JM, Lowenstein DH. Differential regulation of basic helix-loop-helix mRNSa in the dentate gyrus following status epilepticus. Neuroscience 2001;81:10679-10688.
9. Edmondson DG, Lyons GE, Martin J, Olson E. MEF2 gene expression markers the cardiac and skeletal muscle lineages during mouse embryogenesis. Development 1994;20:1251-1263.
10. Leifer D, Kranic D, Yu YT. MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex. PNAS 1993;90: 1546-1550.
11. Yoon SC, Ahn YM, Jun SJ, et al.. Region-specific phosphorylation of ERK5-MEF2C in the rat frontal cortex and hippocampus after electroconvulsive shock. Prog Neuropsychopharmacol Biol Psychiatry 2005; 29:749-753.
12. Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 1996;16:2027-2033.
13. Black BL, Ligon KL, Zhang Y, Olson EN. Cooperative transcriptional activation by the neurogenic basic helix-loop-helix protein MASH1 and members of the myocyte enhancer factor-2 (MEF2) family. J Biol Chem 1996;271:26659-26663.
14. Sloviter RS, Dean E, Sollas AL, Goodman JH. Apoptosis and necrosis induced in different hippocampal neuron populations by repetitive perforant path stimulation in the rat. J Comp Neurol 1996;36:6516-6533.
15. Simonian NA, Getz RL, Leveque JC, Konradi C, Coyle JT. Kainate induces apoptosis in neurons. Neuroscience 1996;74:675-683.
16. Ekdahl CT, Mohapel P, Elmer E, Lindvall O. Caspase inhibitors increase short-term survival of progenitor-cell progeny in the adult rat dentate gyrus following status epilepticus. Eur J Neurosci 2001 4:937-945.
17. Okamoto S, Krainc D, Sherman K, Lipton SA. Antiapoptotic role of the p38 mitogen-activated protein kinase-myocite enhancer factor 2 transcription factor pathway during neuronal differentiation. PNAS 2000; 99:7561-7566.
18. Kikuchi M, Tenneti L, Lipton SA. Role of p38 mitogen-activated protein kinase in axotomy-induced apoptosis of rat retinal ganglion cells. J Neurosci 2000;20:5037-5044.
19. Okamoto S, Li Z, Ju C, et al. Dominant-interfering forms of MEF2 generated by caspase cleavage contribute to NMDA-induced neuronal apoptosis. PNAS 2002;99:3974-3979.