Glutamic acid decarboxylase (GAD)\textsubscript{67}, but not GAD\textsubscript{65}, is constitutively expressed during development and transiently overexpressed by activity in the granule cells of the rat

Jasmín Maqueda\textsuperscript{1}, Mónica Ramírez\textsuperscript{1}, Mónica Lamas\textsuperscript{2}, Rafael Gutiérrez\textsuperscript{*}

Centro de Investigación y Estudios Avanzados del IPN, Apartado Postal 14-740, 07000 Mexico City, D.F., Mexico

Received 17 June 2003; received in revised form 18 August 2003; accepted 29 August 2003

Abstract

\(\gamma\)-Aminobutyric acid (GABA)-mediated neurotransmission from the granule cells to CA3 is transiently expressed during the first 3 weeks of age in the rat. In the adult, seizures provoke this inhibitory signaling to reappear. To gain insight into the origin of GABA in these cells, we explored the expression of both isoforms of glutamic acid decarboxylase (GAD, 65 and 67 kDa), during development and after seizures in the adult rat. We found that GAD\textsubscript{67}, but not GAD\textsubscript{65}, is expressed in the mossy fibers of developing rats. In adults, GAD\textsubscript{67} is no longer detectable, unless seizures are induced. By contrast, GAD\textsubscript{65} is neither expressed in granule cells nor in their mossy fibers at any age nor after seizures, despite the presence of GAD\textsubscript{65} mRNA, confirmed by reverse transcription–polymerase chain reaction in situ.

© 2003 Published by Elsevier Ireland Ltd.

Keywords: Granule cell; Mossy fibers; \(\gamma\)-Aminobutyric acid; Glutamic acid decarboxylase; Development; Seizure
control group for comparison purposes. Hippocampal slices of young rats at the different ages were also simultaneously processed. Immunoreactivity was analyzed in hippocampal slices of four animals of each age group, in four control and four kindled adults. Immunohistology for GAD67 in developing rats was conducted as described [6] using a commercial GAD67 antibody (Chemicon; 1:1000) and a second IgG coupled to FITC (Vector Fl-1000; 1:400). Selected sections were analyzed (usually 14 optical sections of 1 μm) with a scanning confocal microscope (MRC 600, Bio-Rad). Images were acquired with an excitation wavelength of 488 nm (Confocal Assistant; Tood Clark Brelje). For control purposes, each experiment included a slice processed in the absence of the primary antibody. In adult rats, immunohistology for both GAD isoforms was conducted as previously described [13]. For GAD65 experiments, we used the GAD65 antibody purchased from Boehringer Manhein (1:100) and a secondary biotinylated IgG (1:1000; Jackson ImmunoResearch) The slices were processed with the ABC Kit (Vectastain, Vector Labs), developed with diaminobenzidine (Sigma) and contrasted with nickel sulfate (30%). Microscopical observations and micrographs were done with an Axiovert Zeiss microscope with the appropriate photographic equipment.

RT-PCR in situ was conducted in hippocampal slices of three control and three kindled adult rats. It was carried out following previously described protocols [11,12] using previously reported specific GAD65 primers [15]. Tissue samples fixed in paraformaldehyde (4%) were permeabilized with Triton X-100 (0.25%) to insure full penetration of reagents into the tissue and thereafter incubated with DNase I (RNase-free 776785, Roche). We carried out the in situ RT-PCR in the presence of dUTP coupled to digoxigenin with the GeneAmp EZ rTth RNA PCR kit (Applied Biosystems) following instructions from the manufacturer. cDNA sequences were amplified for 30 cycles. After amplification, the tissue was rehydrated, permeabilized and incubated with a digoxigenin antibody (Roche, 1:100) coupled to alkaline phosphatase. The development reaction was carried out with NBT and BCIP (Roche). The processed tissue was analyzed with an inverted microscope (Axiovert-100, Zeiss) and photographed with a digital camera (Sony Cyber Shot DSC-S75). Control experiments comprised the full procedure without either oligonucleotides or anti-digoxigenin antibody or DNase.

As expected, in slices of 10-, 15- and 20-day-old rats, we found GAD67 and GAD65-immunoreactive (-IR) interneurons in all the hippocampal regions. We confirmed the expression of GAD67-IR in the MF of young rats. In Fig. 1A, we show a representative confocal image of the MF region at 15 days of age, where clear immunoreactivity is observed along the stratum lucidum. In contrast, in rats of 1 and 2 months of age, MF did not present GAD67-IR (Fig. 1B). Interestingly, the supposedly responsible enzyme for the synthesis of releasable GABA, GAD65, was neither detected in the GABA-releasing MF of developing rats nor in adult preparations (Fig. 1C,D). We then compared the expression of both isoforms of GAD in control versus kindled epileptic adult rats. We found that GAD67 is constitutively expressed in basket cells and in some granule cells of non-stimulated rats (Fig. 2A), whereas GAD65 was only found in basket cells and other interneurons of the hilus and molecular layer (Fig. 2D). In kindled epileptic rats a marked expression of GAD67 in the granule cells (Fig. 2B) and MF (Fig. 2C) was found besides its overexpression in the interneuronal population. In contrast, GAD65 was absent in granule cells

![Fig. 1. GAD67 is expressed in the mossy fiber pathway of developing (A, 15 days old) but not in adult (B) rats. Notice immunoreactivity along the mossy fibers in the stratum lucidum (SL), in A, and its absence in the adult (B). By contrast, GAD65 immunoreactivity is observed in interneurons (asterisks) but not in the mossy fibers, either in young (C, 15 days old) or in adult rats (D). PCL, pyramidal cell layer; H, hilus. Calibration bars correspond to 100 μm in A,B, and 200 μm in C,D.](image1)

![Fig. 2. Kindling epilepsy up-regulates the expression of GAD67 but not GAD65. (A) The granule cell layer and hilar region present some GAD67-IR granule cells (arrows) and interneurons (asterisks) in control condition. (B) In kindled rats, GAD67 is markedly overexpressed in the granule cell layer (GCL) and in the mossy fibers (C), running along the stratum lucidum (SL). (D) By contrast, in control animals GAD65 is expressed only in interneurons of the molecular (ML) and granule cell layers (GCL) of the dentate gyrus and hilus, although a few cells in the GCL resembling granule cells were GAD65-IR (arrows). Kindling epilepsy does not affect the expression of GAD65 either in the granule cells (E) or in their mossy fibers along the SL. (F) RT-PCR in situ experiments show that despite the lack of the protein, GAD65 mRNA is present in the granule cells of control (G) and kindled rats (H). (I) Control RT-PCR in situ experiment carried out in the absence of the specific oligonucleotides. GAD65 mRNA was not detected under this condition. H, hilus. All calibration bars correspond to 25 μm.](image2)
To determine whether the absence of GAD65 in granule cells in these animals is synthesized by the 67 kDa isoform. Evidence suggests that GABA released from the granule cells inescence technique.

M. Morales for his help in establishing the immunofluorescence technique. The Zeiss microscope used in our study was kindly donated by World Academy of Sciences, Grant 01-407 to R.G. The Ciencia y Tecnología, Grant 36178-N and by the Third World Academy of Sciences, Grant 01-407 to R.G. The Zeiss microscope used in our study was kindly donated by the Alexander von Humboldt Foundation. We thank Dr M. Morales for his help in establishing the immunofluorescence technique.

It is interesting that GAD$_{65}$ mRNA is present in the granule cells of adult control rats, in which no MF GABAergic transmission is observed, and GAD$_{65}$ protein is absent in developing and epileptic rats, where MF GABAergic transmission is active. We confirmed that GAD$_{65}$ is expressed in the MF of developing rats and its expression is down-regulated with age. The induction of seizures and synaptic strengthening up-regulates its expression in the adult dentate gyrus and their MF, which normally contain traces of GABA and transiently express glutamic acid decarboxylase (GAD)$_{65}$ and its mRNA [4,9,13,16–18].

On the contrary, GAD$_{65}$, despite being present during development and up-regulated in interneurons by activity in the adult rat, was not detected either during development or after seizures in the granule cells and their MF. GAD$_{65}$ mRNA was present in granule cells of adult rats but, apparently, it was not modified by seizures. However, to be certain that GAD$_{65}$ mRNA is or is not modulated by seizures, a quantitative method such as real-time PCR has to be used. Thus, we conclude that despite GAD$_{65}$ mRNA being present in the granule cells, it is neither developmentally regulated nor over-expressed by seizures. By contrast, GAD$_{65}$ is normally expressed in developing granule cells, down-regulated after the completion of development and thereafter up-regulated in an activity-dependent fashion. The immunohistochemical evidence gathered so far (present results, [6,13,17,18]), our present in situ RT-PCR results and neurochemical data [2,10] establish that GABA released from the MF is synthesized by the 67 kDa isoform of the enzyme.

Acknowledgements

This work was supported in part by Consejo Nacional de Ciencia y Tecnología, Grant 36178-N and by the Third World Academy of Sciences, Grant 01-407 to R.G. The Zeiss microscope used in our study was kindly donated by Alexander von Humboldt Foundation. We thank Dr M. Morales for his help in establishing the immunofluorescence technique.

References