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Pro-apoptotic function of GABA-related transcripts following stroke

Nadine Jaenisch [a,1], Anke Popp [a,1], Madlen Guenther [a], Juliane Schnabel [a], Otto W. Witte [a,b], Christiane Frahm [a,*]

[a] Hans Berger Department of Neurology, Jena University Hospital, Friedrich Schiller University, Erlanger Allee 101, 07747 Jena, Germany
[b] CSCC, Center for Sepsis Control and Care, Erlanger Allee 101, 07747 Jena, Germany

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ABSTRACT

Following cerebral injuries such as stroke, a structural and functional reorganization of the impaired tissue occurs, which is often accompanied by a re-expression of developmental genes. During brain development, embryonic splice variants of the GABA-synthesizing *GAD67* gene (collectively termed EGAD) participate in cell proliferation, migration, and neuronal differentiation. We thus hypothesized an involvement of EGAD in post-ischemic plasticity. EGAD transcripts were up-regulated at early reperfusion times in the injured area following transient middle cerebral artery occlusion (with a peak expression of 4.5-fold at 6 h in C57BL/6 mice). Cell-specific analysis by a combination of radioactive in situ hybridization and immunolabeling revealed EGAD up-regulation in TUNEL-positive neurons. This unexpected cell death-associated expression of EGAD was confirmed in cell culture models of ischemia (combined oxygen–glucose deprivation) and apoptosis (staurosporine). Staurosporine-mediated cell death led to cleaved Caspase-3 activation, a key regulator of apoptosis following stroke. Blocking of staurosporine-associated EGAD expression via antisense RNA treatment reduced cleaved Caspase-3 activation by ~30%. In addition to the involvement of EGAD in proliferative processes during brain development, we found here that EGAD participates in cell death under pathophysiological conditions in the adult brain. Re-expression of EGAD in neurons following stroke may play a role in aberrant cell cycle activation, consequently being pro-apoptotic. Our observation of a new GABA related pro-apoptotic mechanism and its successful modification might be of significant clinical relevance.

Introduction

The expression of GAD67, one isoform of GABA synthesizing enzymes GAD, is developmentally regulated. In the embryonic brain, alternative transcripts of the GAD67 gene, I-80 and I-86 (collectively termed EGAD), are mainly expressed in regions associated with cell proliferation and migration (Behar et al., 1994; Somogyi et al., 1995). Both I-80 and I-86 code for a short, enzymatically inactive GAD protein (GAD25) with putative regulatory functions (Szabo et al., 1994). I-80 additionally codes for an enzymatically active protein of 44 kDa (GAD44). Around the time of birth, EGAD is down-regulated, whereas the expression of adult GAD67 transcripts increases (Aggensteiner and Reiser, 2003; Greif et al., 1991; Popp et al., 2009b). Although it is known that GAD67 synthesizes GABA for general metabolic processes (for review see Soghomonian and Martin, 1998), the functions of EGAD are currently unclear. However, a persistent expression of EGAD in regions of the adult brain associated with neurogenesis and migration (subventricular zone and rostral migratory stream) as well as with high synaptic plasticity (olfactory bulb) has been demonstrated by our group as well as by others (Krizbai et al., 2000; Popp et al., 2009b).

Cerebral injuries like stroke and epilepsy result in adaptive processes that contribute to the structural and functional reorganization of adjacent and remote brain tissue. Following focal cerebral ischemia, an increase in cell proliferation is observed in neurogenic niches. Newly generated cells migrate to damaged areas where they differentiate into mature neurons (for review see Kernie and Parent, 2010; Zhang et al., 2005). Moreover, synaptic plasticity allows for the integration of new neurons and promotes the rewiring of circuits in perilesional regions (for review see Murphy and Corbett, 2009). In line with our findings that EGAD is expressed in neurogenic niches as well as in regions of high synaptic plasticity in the adult brain (Popp et al., 2009b), it is conceivable that EGAD participates in post-ischemic tissue reorganization. Indeed, an ischemia-induced re-expression of developmental-specific genes (e.g., nestin, MAP2c, and cyclin D1) which corroborates this assumption has been reported (Cramer and Chopp, 2000). Our hypothesis is further supported by the fact that a re-expression of EGAD, followed by an up-regulation of GAD67, has already been described in a model

Abbreviations: as, antisense; cOGD, combined oxygen–glucose deprivation; DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethylsulfoxid; EGAD, embryonic splice variants of GAD67; GABA, gamma-aminobutyric acid; GAD67, glutamic acid decarboxylase 67; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Hmbs, hydroxymethylbilane synthase; MCAO, middle cerebral artery occlusion; qPCR, quantitative PCR; RISH, radioactive in situ hybridization; STS, staurosporine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

* Corresponding author at: Hans Berger Department of Neurology, Jena University Hospital, Erlanger Allee 101, 07747 Jena, Germany. Fax: +49 3641 9 325902.
E-mail address: christiane.frahm@med.uni-jena.de (C. Frahm).

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[1] These authors contributed equally to this work.

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of experimentally induced epileptic seizures (Szabo et al., 2000), which can be interpreted as a mechanism of post-injury plasticity and neuronal protection.

The present study investigated the potential re-expression of EGAD following transient occlusion of the middle cerebral artery in rodents and revealed a robust EGAD expression at early reperfusion times in the ischemic hemisphere. Unexpectedly, EGAD expression was allocated to cells undergoing cell death. The cell death-related EGAD expression was confirmed in in vitro cell culture models of ischemia and apoptosis. Blocking of EGAD re-expression significantly reduced cleaved Caspase-3 activation a key regulator of apoptosis following stroke and therefore revealed a new GABA related pro-apoptotic mechanism.

Materials and methods

Focal stroke model — transient MCAO

All animal procedures were approved by the local government (Thueringer Landesamt, Bad Langensalza, Germany) and conformed to international guidelines on the ethical use of animals. Transient MCAO was induced in ~3-month-old, male Wistar rats and C57Bl/6 mice as previously described in detail (Jaenisch et al., 2010; Sieber et al., 2010). Briefly, by introducing a monofilament into the internal carotid artery, the middle cerebral artery was unilaterally occluded. After 30 min, the suture was withdrawn to restore the blood flow. Sham animals underwent the same surgical procedure as the treatment group, except occlusion of the middle cerebral artery was omitted. Brains were removed at various reperfusion times (2 h, 6 h, 1 d, 7 d).

qPCR following MCAO

Brains were removed following cervical dislocation and coronal sections within the ischemic area were dissected from the cerebrum using a Precision Brain Slicer (Braintree Scientific, Inc.). Coronal sections including the infarct core (rats: +1.0 to −2.0 mm to bregma; mice: +0.8 to −1.2 mm to bregma) were separated into the ipsi- and contralateral parts and snap frozen. Adjacent sections were used for validation of the infarcts as previously described (Popp et al., 2009a; Sieber et al., 2010). Total RNA was isolated using the RNeasy Lipid Tissue Kit (Qiagen) and quantified spectrometrically (ND-1000; NanoDrop). For qPCR experiments, total RNA was transcribed into cDNA using the RevertAid cDNA Synthesis Kit (Fermentas) and then further processed in a 20-μl amplification mixture containing Brilliant® II SYBR Green® qPCR Master Mix (Stratagene), 5 μl cDNA (equivalent to 25 ng reverse transcribed RNA), and 5 μl gene-specific primer mix (500 nmol/l). The qPCR was performed on the Rotorgene 6000 (Corbett Life Science) using gene-specific primers (Table 1, Jaenisch et al., 2010; Sieber et al., 2010). The relative expression level of the ipsilateral vs. the contralateral hemisphere was calculated using the Pfaffl equation and both Gapdh and Hmbs were used as reference genes (Pfaffl, 2001). Four individual rodents per reperfusion time (2 h, 6 h, 24 h, 7 d) were analyzed (rats, n = 16; mice, n = 16).

Table 1
Gene-specific primers for qPCR.

mRNA	Accession number	Sequence 5′ → 3′
		qPCR of rat brain samples
GAD67	NM_017007	fw_GCTGGAAGGCATGGAAGGTTTTA*
		rev_ACGGGTGCAATTTCATATGTGAACATA*
		fw_GCTGGAAGGCATGGAAGGTTTTA*
EGAD	M38350.1	rev_TGAGCCCCATCACCGTAGCA*
		qPCR of mouse brain and cell culture samples
GAD67	NM_008077	fw_ GCTGGAAGGCATGGAAGGCTTTA
		rev_ACGGGTGCAATTTCATATGTGAACATA*
EGAD	Z49977	fw_ GCTGGAAGGCATGGAAGGCTTTA
		rev_TGAGCCCCATCACCGTAGCA*

* Sequences were obtained from Szabo et al. 2000.

In situ hybridization

RISH was carried out according to Popp et al. (2009b) on rats (n = 3). EGAD antisense oligonucleotide probes as well as their sense controls (Popp et al., 2009b; Szabo et al., 2000) were 3′-end labeled with [^{33}P]-dATP (3000 Ci/mmol; Hartmann Analytic GmbH). Frozen coronal rat brain sections (12-μm thickness) were fixed, acetylated, dehydrated, and air dried. Following hybridization for 18 h at 37 °C, slides were washed, dehydrated, coated with Amersham Hypercoat Emulsion LM-1 (GE Healthcare), and exposed for ~6 weeks. After development with Kodak D-19 Developer (Sigma-Aldrich), sections were stained with thionine.

Immunohistochemistry and TUNEL staining

For triple staining, coronal rat brain cryostat sections were fixed and incubated in a blocking solution before the antibodies (rabbit anti-GFAP 1:500, Synaptic Systems; mouse anti-NeuN 1:500, Millipore) were applied in TBS containing 3% normal donkey serum and 0.2% Triton X-100 at 4 °C overnight. Sections were further processed using fluorescence-labeled secondary antibodies (Rhodamine-donkey anti-rabbit, Cy5-donkey anti-mouse, both 1:250; Molecular Probes/Invitrogen). Subsequently, TUNEL staining was applied using the In Situ Cell Death Detection Kit (Roche). Sections were analyzed via confocal laser scanning microscopy (LSM 710; Carl Zeiss MicroImaging GmbH).

To combine RISH with immunohistochemistry or TUNEL staining, sections were incubated with either NeuN or GFAP antibodies, or TUNEL staining solution (as described above) before coating them with the photographic emulsion. Sections were then analyzed microscopically (Axioskop; Carl Zeiss MicroImaging GmbH). Merged images showing co-localization of the RISH signal with the fluorescent signal were produced using ImageJ (NIH). Quantification of co-localized cells was performed in four areas (0.2 mm^2) within the ischemic region (striatum) taking 2 sections from 3 rats, respectively.

Neurosphere culture

Isolation of subventricular cells for neurosphere culture was performed according to Berninger et al. (2007) with minor modifications. The brains of 1-day-old C57Bl/6 mice were removed after cervical dislocation and the lateral wall of the lateral ventricle was dissociated in a hyaluronic acid–trypsin solution (n = 3 per approach). After several washing steps, cells (2.5×10^4/ml) were resuspended in neurosphere medium (containing DMEM and Ham's F12, 1:1; Invitrogen), 1× penicillin/streptomycin (Sigma), 1× B27 supplement (Invitrogen), and 8 mmol/l HEPES (PAA) and seeded into cell culture flasks. Growth factors were added (10 ng/ml EGF and bFGF; Biochrom) every 2nd day to induce neurosphere formation. Neurospheres were passaged every 5th–6th day. To induce differentiation, 6-day-old neurospheres were transferred into poly-D-lysine-coated cell culture plates containing neurosphere medium without growth factors. Neurospheres that had been differentiated for 6 d were used for all experiments. Data were obtained from at least three independent experiments (different preparations of tissue from the subventricular zone).

cOGD

The culture medium of differentiated neurospheres was replaced with glucose-free DMEM and the initial medium was stored. Plates were placed into a humidified chamber (MIC-101; Billups-Rothenberg, Inc.) with subsequent application of an anaerobic gas mixture (95% N_2, 5% CO_2). The chamber was then placed into an incubator at 37 °C. After 2 h, cultures were removed from the chamber and the stored culture medium was re-added. At 2, 6, and 24 h of reperfusion, cells for RNA isolation were collected in Qiazol (Qiagen).