

Cleavage of the Vesicular GABA Transporter under Excitotoxic Conditions Is Followed by Accumulation of the Truncated Transporter in Nonsynaptic Sites

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GABA is the major inhibitory neurotransmitter in the CNS and changes in GABAergic neurotransmission affect the overall activity of neuronal networks. The uptake of GABA into synaptic vesicles is mediated by the vesicular GABA transporter (VGAT), and changes in the expression of the transporter directly regulate neurotransmitter release. In this work we investigated the changes in VGAT protein levels during ischemia and in excitotoxic conditions, which may affect the demise process. We found that VGAT is cleaved by calpains following excitotoxic stimulation of hippocampal neurons with glutamate, giving rise to a stable truncated cleavage product (tVGAT). VGAT cleavage was also observed after transient middle cerebral artery occlusion in mice, a cerebral ischemia model, and following intrahippocampal injection of kainate, but no effect was observed in transgenic mice overexpressing calpastatin, a calpain inhibitor. Incubation of isolated cerebrocortical synaptic vesicles with recombinant calpain also induced the cleavage of VGAT and formation of stable tVGAT. Immunoblot experiments using antibodies targeting different regions of VGAT and N-terminal sequencing analysis showed that calpain cleaves the transporter in the N-terminal region, at amino acids 52 and 60. Immunocytochemistry of GABAergic striatal neurons expressing GFP fusion proteins with the full-length VGAT or tVGAT showed that cleavage of the transporter induces a loss of synaptic delivery, leading to a homogeneous distribution of the protein along neurites. Our results show that excitotoxicity downregulates full-length VGAT, with a concomitant generation of tVGAT, which is likely to affect GABAergic neurotransmission and may influence cell death during ischemia.

Introduction

Glutamate is the major excitatory neurotransmitter in the CNS and together with GABA, the major inhibitory neurotransmitter, controls the activity of neuronal networks. Excitotoxicity mediated by overactivation of glutamate receptors plays a key role in neuronal death characteristic of different disorders of the nervous system, including ischemia, epilepsy, and neurodegenerative diseases (Szydłowska and Tymianski, 2010). The research in cerebral ischemia and excitotoxic neuronal damage has been mainly focused on the excitatory mediators and much less is known regarding the changes in GABAergic activity (Schwartz-Bloom and Sah, 2001). The release of GABA in the ischemic brain and the consequent activation of GABA_A receptors may be neuroprotective through reduction of membrane depolarization. However, Cl⁻ entry through GABA_A receptors in association with

overactivation of glutamate receptors may further increase the influx of water and cell swelling. The former mechanisms may be dominant since neuroprotective strategies to increase GABAergic neurotransmission, targeting both sides of the synapse, have been tested, and some of them were found to be quite efficacious in animal models of ischemia (Schwartz-Bloom and Sah, 2001).

The impairment of GABAergic synaptic transmission in brain ischemia is partly due to a downregulation of synaptic GABA_A receptors, which may contribute to the ongoing neuronal excitability and possibly to neuronal death (Schwartz-Bloom and Sah, 2001). Furthermore, exposure of hippocampal slices to oxygen- and glucose-deprivation was shown to induce an early release of GABA by exocytosis, followed by a delayed phase of neurotransmitter release mediated by reversal of the plasma membrane transporter (Allen et al., 2004). However, few studies have addressed the mechanisms underlying the presynaptic changes in GABAergic synapses. Much evidence has been gathered showing that the vesicular neurotransmitter transporters can influence the neural transmission, and changes in expression of the vesicular transporters have been shown to alter the exocytosis of amino acid neurotransmitters (Wilson et al., 2005; Erickson et al., 2006; Edwards, 2007; Wu et al., 2007; Lima Rde et al., 2010). Therefore, putative changes in the levels of vesicular GABA

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transporter (VGAT) in the ischemic brain may influence the release of GABA and therefore the balance between excitatory and inhibitory activity. In a recent work, the upregulation of VGLUT 1 was correlated with an increase in glutamatergic transmission, contributing to an increase in extracellular glutamate levels and hyperexcitability in patients with temporal lobe epilepsy (van der Hel et al., 2009).

Calpains are cysteine proteases involved in ischemic/excitotoxic cell death and in several chronic neurodegenerative conditions (Bevers and Neumar, 2008; Vosler et al., 2008). Under normal physiological conditions the activation of calpains is tightly regulated and plays a key role in the cleavage of selected protein targets (Liu et al., 2008). Activation of these proteases due to a $[Ca^{2+}]_i$ overload under excitotoxic conditions cleaves key synaptic proteins in glutamatergic synapses, including ionotropic (AMPA and NMDA receptor subunits) and metabotropic glutamate receptors (Xu et al., 2007; Yuen et al., 2007; Gascón et al., 2008), and postsynaptic scaffold proteins (Jourdi et al., 2005), but little information is available regarding the effect of calpains on GABAergic synapses. In the present study we evaluated the changes in VGAT protein levels and cellular distribution in pathological conditions, including excitotoxicity, brain ischemia, and following status epilepticus. The results show that VGAT is cleaved into a new stable truncated form that is not targeted to synapses, which is likely to affect GABAergic synaptic transmission.

Materials and Methods

Primary neuronal cultures

Cultures of rat hippocampal neurons were prepared from embryonic day 18 (E18) Wistar rat embryos as described previously (Almeida et al., 2005; Caldeira et al., 2007). Cortical and striatal neurons were cultured using the same procedures, from E15–16 Wistar rat embryos. Neuronal cultures were maintained in serum-free Neurobasal medium (Invitrogen), supplemented with B27 (Invitrogen), glutamate (Sigma, 25 μ M), glutamine (Sigma, 0.5 mM), and gentamicin (Invitrogen, 0.12 mg/ml). Striatal and cortical cultures were maintained in the absence of glutamate. The cells were kept at 37°C in a humidified incubator with 5% CO₂/95% air, for 7 d (hippocampal cultures) or 14–15 d (cortical and striatum cultures). Cells were cultured at a density of 90 000 cells/cm² or 80 000 cells/cm² on poly-D-lysine-coated (Sigma) six-well microplates (MW6) (for Western blot and real-time PCR experiments) or glass coverslips (for immunocytochemistry studies), respectively.

PC12 cell line

Pheochromocytoma PC12 cells were cultured in DMEM/F12 (1:1) (Invitrogen), supplemented with NaHCO₃, pH 7.2, 5% fetal bovine serum (Invitrogen), 10% horse serum (Invitrogen), and 1% penicillin/streptomycin (Sigma). Cells were diluted every 2–3 d after achieving 80–90% confluency.

Western blot

Hippocampal, cortical, and striatal neuron cultures were washed twice with ice-cold PBS and once more with PBS buffer supplemented with 1 mM DTT and a mixture of protease inhibitors (0.1 mM PMSF, CLAP: 1 μ g/ml chymostatin, 1 μ g/ml leupeptin, 1 μ g/ml antipain, 1 μ g/ml pepstatin; Sigma). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC, and 0.1% SDS at a final pH 7.5) supplemented with the mixture of protease inhibitors. After centrifugation at 16,100 \times g for 10 min, protein in the supernatants was quantified using the BCA method, and the samples were denatured with 2 \times concentrated denaturing buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue). Extracts used for VGAT analysis were not subjected to a boiling step because it leads to VGAT aggregation.

Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Milli-

pore), and immunoblotted, as described previously (Caldeira et al., 2007). Blots were incubated with primary antibodies (overnight at 4°C), washed, and exposed to alkaline phosphatase-conjugated secondary antibodies [1:20,000 dilution for anti-rabbit IgG and 1:10,000 dilution for mouse IgG (GE Healthcare); 1 h at room temperature]. Alkaline phosphatase activity was visualized by enhanced chemifluorescence on the Storm 860 gel and blot imaging system and quantified using the ImageQuant program (GE Healthcare). The following primary antibodies were used: anti-VGAT (1:2000, Ab: 75–87 aa; Synaptic Systems), anti-VGAT (1:1000, Ab: 1–20 aa; Sigma), anti-VGAT (1:500, Ab: 500–525 aa; Millipore), anti-NR1 (1:200, Millipore), anti-human calpastatin (1:5000, Takara), and anti-GFP (1:2000, Abcam). The anti-synaptophysin (1:20,000, Synaptic Systems) antibody was used as loading control.

In control experiments the specificity of the anti-VGAT antibody (amino acids 75–87; 1:2000) was tested after preabsorbing the antibody with the peptide used for immunization (1 μ g/ml; Synaptic Systems). The preadsorption was performed overnight at 4°C before incubation with the blotting membrane.

Immunocytochemistry

Cells were fixed in 4% sucrose/paraformaldehyde and permeabilized with 0.3% Triton X-100 in PBS. The neurons were then incubated with 0.2% gelatin (Sigma) in PBS + 0.1% Tween 20, for 1 h at 37°C, to block nonspecific staining, and incubated with primary antibodies, overnight at 4°C. The cells were then washed five times with PBS + 0.1% Tween + 0.1% gelatin and incubated with the appropriate secondary antibodies, for 1 h at 37°C. The coverslips were mounted in a fluorescent mounting medium (DAKO), and imaging was performed on a Zeiss LSM510 confocal microscope, using a 63 \times oil objective. The primary antibodies used were anti-VGAT (1:500, Ab: 75–87 aa; Synaptic Systems), anti-GABA (1:200, Abcam), and anti-synaptophysin (1:200, Sigma). The secondary antibodies used were Alexa Fluor 488, 594, 647, and Texas Red (Invitrogen). The fluorescent dye Hoechst 33342 (Sigma, 0.5 μ g/ml) was used to stain nuclei.

mRNA semiquantification through real-time PCR

Total RNA was extracted from 7 d *in vitro* (DIV) cultured hippocampal neurons using TRIzol Reagent (Invitrogen), as described previously (Santos and Duarte, 2008), and the RNA quality and integrity was assessed using the Experion automated gel-electrophoresis system (Bio-Rad), as described previously (Santos and Duarte, 2008). Samples showing RNA degradation or contamination by DNA were discarded. RNA concentration was determined using the fluorescent dye RiboGreen (Invitrogen-Molecular Probes) or NanoDrop 1000 (Thermo Scientific). The samples were aliquoted and stored at –80°C until further use. cDNA synthesis was performed using 2 μ g of total RNA and the AMV reverse transcriptase (10 U; Roche), as described previously (Santos and Duarte, 2008). Samples were stored at –80°C until further use.

Primers for real-time PCR were designed using the “Beacon Designer 7” software (Premier Biosoft International) as described previously (Santos and Duarte, 2008). The primers (Fisher Scientific) used for VGAT were as follows: forward, 5′GCCGTGTCCAAGTTCAGTC3′ and reverse, 5′AGAGACAGTAGGCGATGACC3′. *Gapdh* was used as reference gene with the following primers: forward, 5′CTCCCATCTCCACCTTTG3′ and reverse, 5′TGTAGCCATATTCATTGTCATACC3′. The annealing temperature was 55°C.

For gene expression analysis, 2 μ l of 1:100 diluted cDNA was added to 10 μ l of 2 \times SYBR Green Master Mix (Bio-Rad), and the final concentration of each primer was 250 nM in 20 μ l total volume. The thermocycling reaction was initiated with activation of the TaqDNA Polymerase by heating at 95°C during 30 s, followed by 55 cycles of a 10 s denaturation step at 95°C, a 30 s annealing and a 30 s elongation step at 72°C. The fluorescence was measured after the extension step, using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction, the melting step was performed with slow heating, starting at 55°C and with a rate of 0.5°C per 10 s, up to 95°C, with continuous measurement of fluorescence.

Data analysis was performed using Pfaffl method for efficiency correction (Pfaffl, 2001). The results were normalized with *Gapdh* as internal

reference gene because it showed a stable expression in hippocampal neurons stimulated with glutamate.

DNA constructs

The rat full-length cDNA of VGAT was kindly provided by S. Takamori (Tokyo Medical and Dental University, Tokyo, Japan) (Takamori et al., 2000). The amplified fragment was digested by BamHI and XbaI (New England Biolabs) and purified by gel electrophoresis before subcloning into pcDNA3.1 (Invitrogen) at BamHI/XbaI sites.

Plasmids encoding truncated VGAT lacking amino acids 2–51 or 2–59 [pcDNA3.1-VGATΔ(1–52) and pcDNA3.1-VGATΔ(1–60)], respectively, were generated by site-directed mutagenesis (STRATAGENE). Both truncated proteins have amino acid 1 before the amino acids that correspond to the cleavage site, to ensure the translation of the protein in the cells.

The plasmid encoding the VGAT-GFP fusion protein was generated by molecular cloning. The cDNA of VGAT was fused into the green fluorescent protein (GFP) gene in pEGFP-N1 vector (BD Biosciences Clontech) through KpnI and AgeI sites, using the primers: forward, 5' CGG GGTACC ATGGCCACCCTGCTCCGCAG 3' and reverse, 5' CGG ACCGGT GA GTCCTCTGCGTTGGTTCGGTA 3'. The forward primer has the following: 3 nucleotides at the beginning (CGG) to improve KpnI cleavage (close to the end of DNA fragments), the KpnI cleavage site (GGTACC), and then the starting codon ATG followed by additional nucleotides of VGAT cDNA. The reverse primer has the following: the same 3 nucleotides at the beginning (CGG) to improve AgeI cleavage, the AgeI cleavage site (ACCGGT), two nucleotides (GA) so that GFP stays in frame, the stop codon of VGAT is removed, and then the cDNA of VGAT. The resulting pEGFP-N1-VGAT sequence was verified by DNA sequencing reactions. Immunocytochemistry experiments using antibodies against VGAT and GFP showed that the GFP-VGAT fusion protein has the same cellular localization as VGAT endogenous protein in cultured hippocampal neurons (data not shown). The truncated GFP-VGAT plasmids were generated by site-directed mutagenesis.

The plasmid encoding the fusion protein of mouse N-terminus of VGAT with glutathione S-transferase (GST) was kindly provided by Bruno Gasnier (Université Paris Descartes, Paris, France) (Dumoulin et al., 1999). Briefly, the first 127 aa of the mouse VGAT were cloned with BamHI and EcoRI into the pGEX4T1 vector (Pharmacia), with minor modifications. The resulting plasmid, pGEX4T1-VIAATNter, was verified by automated sequencing (STAB VIDA). The N-terminus of VGAT from rat and mouse differ in only one base. The high homology between the mouse and rat VGAT in the N-terminal region is in agreement with the results showing the cleavage of the transporter in cultured neurons from both species under excitotoxic conditions (data not shown).

Expression of recombinant GST-VGAT N-terminus

Expression of the GST-VGAT N-terminus fusion protein (pGEX4T1-VGAT N-terminus) and GST (pGEX4T1) was induced by treating an *Escherichia coli* BL21 culture in the exponential phase of growth ($A_{600\text{ nm}} = 0.8\text{--}2$) with 0.5 mM isopropyl β -D-thiogalactoside (BIORON) for 30 min at 30°C. The protein, with an apparent molecular mass of 44 kDa in a SDS-PAGE gel, was extracted and purified from the bacterial pellet through affinity chromatography on glutathione Sepharose 4B (GE Healthcare), according to manufacturer's recommendations.

Transfection

Transfected PC-12 cells overexpressing VGAT (pcDNA3.1-VGAT) or the truncated forms of the transporter [pcDNA3.1-VGATΔ(1–52) and pcDNA3.1-VGATΔ(1–60)] were generated using a plasmid DNA vector technique. PC-12 cells were transfected using GenePORTER 2 (Gene Therapy Systems). VGAT and truncated cleavage product (tVGAT) overexpression was confirmed by Western blot. The same approach was used to generate PC-12 cell lines overexpressing GFP-VGAT (pEGFP-N1-VGAT) and GFP truncated VGAT [pEGFP-N1-VGATΔ(1–52) and pEGFP-N1-VGATΔ(1–60)].

Transfection of cultured striatal and hippocampal neurons was performed by the calcium phosphate coprecipitation method as described previously, with minor modifications (Jiang et al., 2004; Kaech and Banker, 2006). Briefly, 2 μ g of plasmid DNA were diluted in Tris-EDTA

(TE) pH 7.3 and mixed with HEPES calcium chloride pH 7.2 (2.5 mM CaCl_2 , 10 mM HEPES). This DNA/TE/calcium mix was added to a 2 \times HEPES-buffered saline solution (270 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4 , 11 mM dextrose, 42 mM HEPES), pH 7.2. The precipitates were allowed to form for 30 min, with vortex mixing every 5 min, to ensure that the precipitates had similar small sizes. Meanwhile, coverslips with cultured neurons were incubated with cultured conditioned medium with 2 mM kynurenic acid (Sigma). The precipitate was added drop-wise to each coverslip and incubated at 37°C, 5% CO_2 , for 3 h. The cells were then washed with acidic 10% CO_2 equilibrated culture medium containing 2 mM kynurenic acid and returned to the 37°C/5% CO_2 incubator for 15 min. Finally, the medium was replaced with the initial culture-conditioned medium, and the cells were further incubated in a 37°C/5% CO_2 incubator for 48 h to allow protein expression.

In vitro calpain assay

Incubation of GST-VGAT N-terminus with calpain. GST-VGAT N-terminus fusion protein (100 μ g) was diluted in 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM dithiothreitol, and 2.5 mM CaCl_2 (or 1 mM EDTA), and incubated with or without 2.5U/ml recombinant calpain I (human erythrocytes, Calbiochem), for 15 min at 37°C. Incubation was stopped with concentrated denaturing buffer and boiled for 5 min at 95°C. The peptides were then separated by SDS-PAGE with tricine gels, to allow separation of low molecular weight proteins (Schägger and von Jagow, 1987). The gel was stained with CommaSSie colloidal solution (Candiano et al., 2004). The same experimental procedure was done to transfer the proteins to a PVDF membrane (Millipore), and the bands were cut and sent to N-terminal Edman sequencing, at the protein sequencing facility in University of Leeds, United Kingdom.

Incubation of synaptic vesicles with calpain. Cerebrocortical synaptic vesicles were isolated from the rat brain as described previously (Synaptic Systems—protocol for preparation of P2 and LP2 fractions). The vesicles (5 μ g) were diluted in 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM dithiothreitol, and 2.5 mM CaCl_2 (or 1 mM EDTA), and incubated with or without 2.5U/ml recombinant calpain I (human erythrocytes, Calbiochem), for different time points at 37°C. Where indicated, the experiments were conducted in media with free calcium concentrations of 100 nM, 500 nM, or 1 μ M, prepared with 0.5 mM EGTA and the CaCl_2 concentrations determined using Maxchelator (<http://www.stanford.edu/~cpatton/webmaxc/webmaxclite115.htm>) (Patton et al., 2004; McGuigan et al., 2007). Incubation was stopped with concentrated denaturing buffer and no boiling step was done to prevent VGAT aggregation. VGAT cleavage was analyzed by Western blot.

In vitro phosphatase assay

In vitro phosphatase assay was done as described previously (Bedet et al., 2000). Total cell extracts (50 μ g) from cultured hippocampal neurons solubilized in RIPA were diluted in the intestinal calf alkaline phosphatase (CIP) buffer, containing 50 mM Tris-HCl, pH 7.9, 10 mM MgCl_2 , 100 mM NaCl, 1 mM dithiothreitol, and supplemented with 0.1 mM PMSF, 1 μ g/ml chymostatin, 1 μ g/ml leupeptin, 1 μ g/ml antipain, 1 μ g/ml pepstatin (Sigma), and 2 U/ml CIP (New England Biolabs). Incubations were performed for 1 h at 37°C and stopped with 2 \times concentrated denaturing buffer. VGAT protein levels were determined by Western blot.

Intrahippocampal injection of kainate

Injection. Intrahippocampal injection of kainate (Tocris Bioscience) was performed as described previously (Tomioka et al., 2002; Takano et al., 2005), with minor modifications. Briefly, wild-type adult male mice (C56BL6) and transgenic mice overexpressing human calpastatin (Higuchi et al., 2005) were deeply anesthetized with avertin (2,2,2-tribromoethanol, 2-methyl-2-butanol), placed in a stereotaxic apparatus (Stoelting), and given a unilateral injection of 0.1 nmol of kainate (in 0.3 μ l of PBS) into the hippocampal CA1 region, using a 10 μ l motorized syringe (Hamilton) after drilling a small hole with a surgical drill. The coordinates of the injection were anterior-posterior -2.3 mm, medial-lateral -1.5 mm, and dorsal-ventral -1.7 mm from the bregma. Two minutes after the needle insertion, kainate was injected at a constant flow rate of 0.05 μ l/min. The needle remained in place for an additional 2 min to prevent reflux of fluid. The body temperature of mice was monitored and maintained at 37°C during surgery and 30 min after the

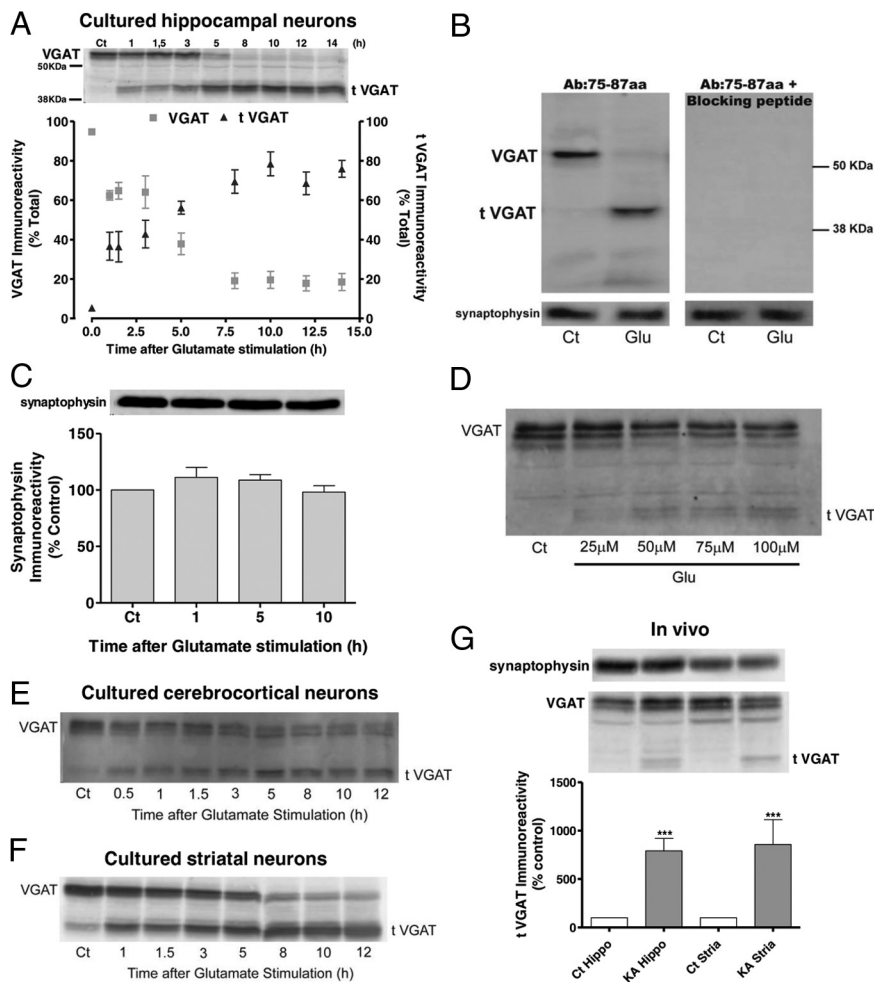


Figure 1. VGAT is cleaved under *in vitro* and *in vivo* excitotoxic conditions giving rise to tVGAT. **A**, Cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate (125 μ M glutamate, 20 min) and further incubated in culture-conditioned medium. VGAT (**A**) and synaptophysin (**C**) protein levels were determined by Western blot at the indicated time points after excitotoxic stimulation. The results are the average \pm SEM of six and four independent experiments, respectively, performed in different preparations. Statistical analysis was performed using one-way ANOVA followed by Dunnett's comparison test performed for each condition as compared with the control. **B**, Extracts from control and glutamate stimulated hippocampal neurons were analyzed by Western blot using an antibody absorbed with the peptide used for immunization. The dose-dependent effect of glutamate on the cleavage of VGAT is shown in **D**. Cultured hippocampal neurons were stimulated with 25–100 μ M glutamate for 20 min and further incubated in culture-conditioned medium for 1.5 h. The results are representative of three experiments. **E** and **F** show the results obtained in similar experiments using cultured cerebrocortical and striatal neurons, respectively. **G** shows the effect of *in vivo* excitotoxicity on the cleavage of VGAT. Adult Wistar rats were injected with kainate (30 mg/kg, i.p.) and 24 h later the hippocampi and striatum were collected. Extracts from both brain regions were analyzed by Western blot. The amount of truncated VGAT and synaptophysin in the hippocampus of control and kainate-injected animals was compared using the one-way ANOVA followed by Newman–Keuls multiple comparison test, for three independent experiments. *** $p < 0.001$, compared with the control.

injection with a homeothermic heating blanket (Harvard Apparatus), with feedback regulation. The death rate in this experiment was $<5\%$. Experiments were conducted according to the European Union Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

Western blot. Twenty-four hours after injection mice were killed and a 2 mm section around the hippocampus was taken with the help of a 1 mm coronal mouse matrix (Harvard Apparatus). The slices were immediately frozen with dry ice and the damaged ipsilateral and contralateral areas of the hippocampal slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for Western blot.

Histochemistry. This was performed as described previously (Takano et al., 2005) with minor modifications. Twenty-four hours after injection of kainate or PBS, mice were deeply anesthetized and perfused through

the heart with ice-cold PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, postfixed in the same fixative overnight at 4°C, and then left in 25% sucrose in PBS at 4°C, until sinking in the solution. Coronal sections (30 μ m) were cut on a freezing microtome (Leica Cryostat CM 3050) and mounted on gelatin-chromium covered glass slides. Sections were then stained with cresyl violet (Sigma) and examined under a Zeiss Axiovert 200 microscope.

Intraperitoneal injection of kainate

Intraperitoneal injection of kainate was performed as described previously (Wang et al., 2005). Adult rat (Wistar) with an average age of 6 months were injected with a sterile saline solution (0.32 M sucrose, 10 mM HEPES, pH 7.4) containing 10 mg/kg kainate (Tocris Bioscience). After injection they were left with food and water *ad libitum*. All the animals used in the present study achieved status epilepticus in the next few hours after injection (Silva et al., 2005). Twenty-four hours after kainate injection the rats were killed and the cerebral cortex and striatum were collected for Western blot analysis. The death rate in this experiment was $<10\%$. All experiments were done in accordance with the European ethical guidelines for the care and use of laboratory animals (86/609/EEC).

Middle cerebral artery occlusion

Focal cerebral ischemia was induced by the transient occlusion of the right middle cerebral artery (MCA), using the intraluminal filament placement technique as described previously (Nygren and Wieloch, 2005). Briefly, adult male mice were anesthetized by inhalation of 2.5% isoflurane (IsobaVet, Schering-Plough Animal Health) in $O_2:N_2O$ (30:70). Anesthesia was subsequently reduced to 1.5–1.8% isoflurane and sustained throughout the occlusion period. Body temperature was kept at $\sim 37^\circ\text{C}$ throughout the surgery period. To monitor regional cerebral blood flow (rCBF), an optical fiber probe (Probe 318-I, Perimed) was fixed to the skull at 2 mm posterior and 4 mm lateral to bregma and connected to a laser Doppler flow meter (Periflux System 5000, Perimed). A filament composed of 6–0 polydioxanone suture (PSD II, Ethicon) with a silicone tip (diameter of 225–275 μ m) was inserted into the external carotid artery and advanced into the common carotid artery. The filament was retracted, moved into the internal carotid artery, and advanced until the origin of the MCA, given by the sudden drop in rCBF ($\sim 70\%$ of baseline). After 45 min, the filament was withdrawn and reperfusion observed. The animals were placed in a heating box at 37°C for the first 2 h after surgery and thereafter transferred into a heating box at 35°C , to avoid postsurgical hypothermia. Thirty minutes after the onset of reperfusion, 0.5 ml of 5% glucose was administered subcutaneously. Temperature and sensorimotor deficits were accessed at 1 and 2 h, and in the morning after the surgery.

The Ethics Committee for Animal Research at Lund University approved animal housing conditions, handling, and surgical procedures. Nine to 11 weeks old C57BL/6J male mice (weight: 21.5 g to 27.9 g; Taconic) were housed under diurnal conditions with *ad libitum* access to water and food before and after surgery.

Mice were killed 6 and 24 h after MCA occlusion (MCAO), and 1 mm sections were done with the help of a 1 mm coronal mouse matrix. The slices were immediately frozen with dry ice and the damaged ipsilateral and contralateral areas of the slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for Western blot as described above.

2,3,5-Triphenyltetrazolium chloride staining

After 24 h of recovery, animals were killed to evaluate the infarct volume. The brain was removed and the forebrain was sliced into 1-mm-thick sections using a mouse brain slicer, on ice. The sections were rinsed once in ice-cold 0.9% sodium chloride (NaCl) for 10 min and subsequently immersed in 50 ml of 0.01% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.9% NaCl at 37°C for 15 min. Slices were fixed in 4% formalin and images were acquired with a MicroPublisher 3.3 RTV charge-coupled device camera (QImaging) using standard conditions.

Chemical long-term potentiation

Cultured hippocampal neurons were exposed to conditions designed to induce chemical long-term potentiation (LTP), as described previously (Shepherd et al., 2006). Briefly, cultured hippocampal neurons with 15 DIV were incubated at 37°C and 5% CO₂ for 15 min in artificial CSF (ACSF) containing 126 mM NaCl, 5.5 mM KCl, 0.4 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM NaHCO₃, and 2 mM CaCl₂, and supplemented with 14 mM dextrose, 0.2 mM glycine, and 0.01 mM bicuculline. After stimulation, the cultures were further incubated in culture-conditioned medium for the indicated time periods.

Statistical analysis

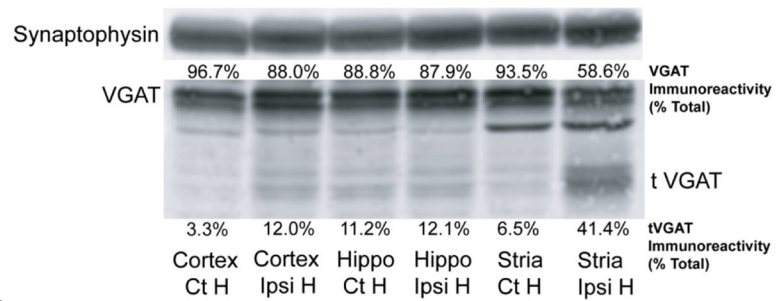
The immunoreactivity obtained in each experimental condition was calculated as a percentage of the control. Data are presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis of the results was performed using one-way ANOVA analysis followed by either the Dunnett's or Bonferroni post-tests.

Results

VGAT is cleaved under excitotoxic conditions giving rise to a truncated cleavage product

To understand how excitotoxic conditions influence the protein levels of the vesicular GABA transporter, cultured neurons isolated from different brain regions were stimulated with glutamate (125 μM, for 20 min) and further incubated in culture-conditioned medium for different periods of time. Stimulation of cultured hippocampal neurons under these conditions induces 40–50% cell death (Almeida et al., 2005), and Western blot experiments, using an antibody against the N-terminal region of VGAT, showed a biphasic cleavage of the transporter, giving rise to a product of ~46 kDa (tVGAT) (Fig. 1A). VGAT protein levels dropped rapidly by ~40% within the first hour after the toxic insult, and a secondary decrease in full-length VGAT protein levels, with the same magnitude, was

A 24h after lesion



B 6h after lesion

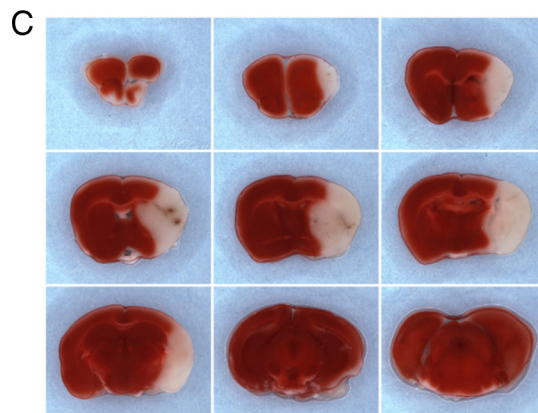
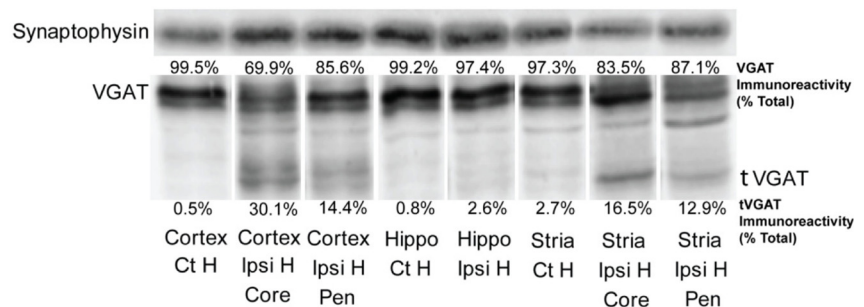


Figure 2. Transient focal ischemia induces VGAT cleavage and tVGAT formation. The changes in VGAT immunoreactivity in the ipsilateral (Ipsi H) and contralateral (Ct H) brain hemispheres of adult mice (C57BL/6J) subjected to a transient 45 min occlusion of the left middle cerebral artery are shown in **A** and **B**. VGAT protein levels in the cerebral cortex, hippocampus (Hippo), and striatum (Stria) were determined by Western blot, 6 h (**B**) or 24 h (**A**) after the lesion. Core and penumbra (pen) regions were dissected separately in the cerebral cortex and striatum. tVGAT and VGAT immunoreactivity is also quantified in **A** and **B**, respectively. **C** shows the TTC staining of 1-mm-thick sections of the mouse brain, 24 h after the occlusion, providing an evaluation of the infarct volume and damaged areas in the brain after the 45 min occlusion of the right middle cerebral artery.

observed at 3–8 h after excitotoxic stimulation. The downregulation of the full-length VGAT was associated with an upregulation of a 46 kDa immunoreactive band, suggesting that this is a cleavage product of the transporter. The full-length and the cleaved transporter were not detected in Western blot experiments with an antibody that had been previously blocked with the peptide used in the immunization (Fig. 1B).

The effects of glutamate stimulation on VGAT cleavage were also tested using different concentrations of the ligand, and the abundance of full-length and cleaved transporter was evaluated 1.5 h after stimulation (Fig. 1D). The results show a cleavage of the transporter even when neurons were stimulated with glutamate at 50 μM, a concentration that does not induce neuronal death (data not shown). Under the excitotoxic conditions that caused VGAT cleavage, the synaptic vesicle protein synaptophysin was not affected (Fig. 1C), showing that the cleavage of VGAT

is not part of a general cascade of protein cleavage. Similar results were obtained in cultured cerebrocortical (Fig. 1E) and striatal (Fig. 1F) neurons, using the same protocol for excitotoxic stimulation.

To determine whether VGAT cleavage also occurs under excitotoxic conditions *in vivo*, VGAT and tVGAT protein levels were analyzed in the hippocampus and striatum of Wistar rats injected with kainate (30 mg/kg, i.p.), an animal model of temporal lobe epilepsy (Goodman, 1998). Systemic injection of kainic acid reproducibly causes cell death in several brain areas in a seizure-dependent process, the hippocampus being the most affected region (Araújo et al., 2008). Twenty-four hours after kainate injection the truncated transporter was found in the hippocampus and striatum, while synaptophysin protein levels were not changed (Fig. 1G).

The formation of VGAT truncation products was also assessed using the MCAO model of transient focal ischemia (Traystman, 2003). Adult mice (C57BL/6J) were subjected to a transient 45 min occlusion of the left middle cerebral artery, and extracts were prepared from the ipsilateral (Ipsi H) and contralateral (Ct H) brain hemispheres (cortex, striatum, and hippocampus) 6 and 24 h after injury. A downregulation of VGAT protein levels associated with an upregulation of tVGAT was observed in the ipsilateral striatum and cerebral cortex 6 h after the ischemic insult (Fig. 2B). This effect was more significant in the core than in the penumbra region, and a significant amount of tVGAT was still present in the damaged (ipsilateral) striatum 24 h after MCAO (Fig. 2A). In contrast, MCAO did not induce cleavage of VGAT in the hippocampus, a brain region that is not affected in this model of ischemia, as observed in 1-mm-thick sections of the mouse brain, 24 h after the occlusion, using TTC staining (Fig. 2C).

VGAT is cleaved under conditions designed to induce chemical LTP

Since low concentrations of glutamate also lead to the cleavage of VGAT (Fig. 1D), we investigated the effects of increased synaptic activity induced by stimulation of the cells with 0.2 mM glycine and 0.01 mM bicuculline for 15 min. This protocol stimulates excitatory synaptic activity and was previously shown to induce chemical LTP (Shepherd et al., 2006). The cells were further incubated in culture-conditioned medium for the indicated periods of time before preparation of extracts. Small but significant amounts of tVGAT were observed 5–8 h after stimulation, when chemical LTP is typically observed (Shepherd et al., 2006), and this effect was also sensitive to the calpain inhibitor ALLN (Fig. 3).

tVGAT has a long half-life

The results of Figure 1A show that excitotoxic stimulation of hippocampal neurons with glutamate causes a time-dependent cleavage of VGAT, giving rise to a truncated product. Interestingly, calculation of the total protein (full-length + truncated VGAT immunoreactivity) expressed in the cells after the insult showed no significant differences for the different incubation periods tested, up to 12 h (Fig. 4A). tVGAT is present even 24 h after the glutamate stimulation (Fig. 4B). These results strongly suggest that truncated VGAT has a high stability in the cell.

The replenishment of the pool of full-length transporters after cleavage depends on the turn-over rate, which can be assessed by blocking translation activity. Incubation of hippocampal neurons with the translation inhibitor anisomycin (Calbiochem; 5 μ M) for 8 h did not change significantly total VGAT protein levels

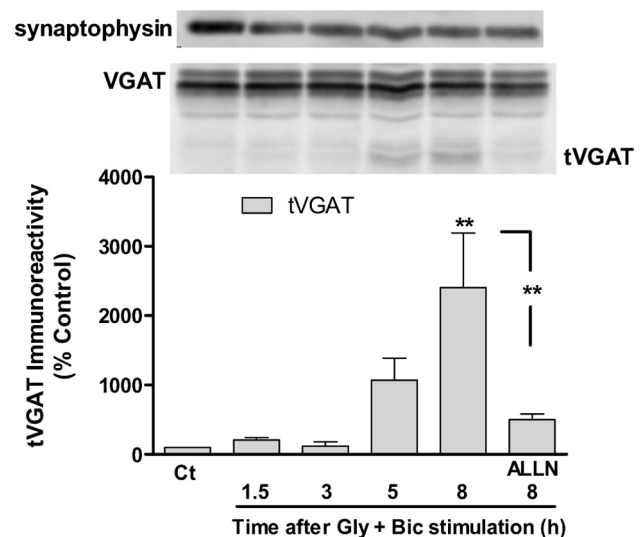


Figure 3. VGAT is cleaved under conditions that induce chemical LTP. Cultured hippocampal neurons (15 DIV) were stimulated with 0.2 mM glycine and 0.01 mM bicuculline for 15 min in ACSF. The cells were then incubated in culture-conditioned medium for the indicated periods of time before preparation of the extracts. Where indicated, the cells were pre-incubated with the calpain inhibitor ALLN (50 μ M) for 2 h, and the inhibitor was also present in the culture-conditioned medium after excitotoxic stimulation. The results were analyzed by Western blot, using an antibody against VGAT. The results are the average \pm SEM of three different experiments performed in independent preparations. Statistical analysis was performed using the one-way ANOVA followed by Newman–Keuls multiple comparison test. $**p < 0.01$, compared with the control or with cells exposed to chemical LTP in the absence of ALLN.

in control conditions (Fig. 4C), showing that the protein has a rather slow turn-over rate. In contrast, inhibition of translation for the same period of time decreased significantly the protein levels of GluN1, a subunit of the NMDA receptors (Fig. 4D). The mRNA for VGAT was also downregulated to some extent under excitotoxic conditions (Fig. 4E). This is likely due to active degradation of VGAT transcripts since inhibition of transcription with actinomycin D (Calbiochem), for the same period of time, did not change significantly VGAT mRNA (Fig. 4E). Together, the results showing a low turn-over rate of VGAT and the high stability of tVGAT indicate that in surviving neurons the truncated protein replaces the full-length transporter for 12–24 h after excitotoxic stimulation.

Calpain cleaves VGAT giving rise to tVGAT, under *in vitro* and *in vivo* excitotoxic conditions

Chemical inhibitors of different classes of proteases (calpains, caspases, cathepsins, and the proteasome) were used to dissect their relative role in VGAT cleavage under excitotoxic conditions. Both calpain inhibitors used, ALLN and MDL28170 (Calbiochem), prevented VGAT cleavage and tVGAT formation mainly in the earlier phase, observed 1.5 h after glutamate stimulation (Fig. 5A). The second and slower phase of VGAT cleavage, observed 2–7 h after glutamate stimulation (see Fig. 1A), was only partially sensitive to calpain inhibition (Fig. 5B). In agreement with the role of calpains in VGAT cleavage, ALLN also prevented the cleavage of the transporter in hippocampal neurons incubated under conditions that induce chemical LTP (Fig. 3).

Since VGAT cleavage was not fully inhibited by calpain inhibitors, we investigated the putative role of caspases and cathepsins in VGAT downregulation. Z-VAD FMK (Biomol), a general inhibitor of caspases, did not affect glutamate-

induced VGAT cleavage (Fig. 5D), indicating that caspases do not contribute to VGAT cleavage under excitotoxic conditions. Cathepsins, mainly the isoform B and L, have been described as being involved in protein degradation in ischemic cell death (Yamashima, 2000). The use of specific pharmacological inhibitors of cathepsin B and L (CA-074 Me and Cathepsin L Inhibitor III, respectively, at 50 and 10 μM , Calbiochem) showed that these proteases do not contribute to the cleavage of VGAT under excitotoxic conditions (Fig. 5E). Furthermore, cathepsin inhibitors did not show a significant effect on VGAT cleavage when tested in the presence of the calpain inhibitor (50 μM). The role of the proteasome in glutamate-induced VGAT cleavage was tested using the inhibitor MG132 (Calbiochem; 1 μM). The effect of the proteasome inhibitor was tested 6 h after excitotoxic stimulation due to its toxicity at longer incubation periods (Rideout and Stefanis, 2002; Butts et al., 2005). MG132 did not affect glutamate-induced VGAT cleavage (Fig. 5F), ruling out the contribution of the proteasome.

To confirm the effect of calpain on VGAT, we performed *in vitro* experiments using isolated cerebrocortical synaptic vesicles and recombinant calpain I (Fig. 5C). The results show a time-dependent cleavage of VGAT in the presence of calpain and the formation of a truncated VGAT with an apparent molecular mass of ~ 46 – 47 kDa, similar to the results obtained in cultured hippocampal neurons (Fig. 1A). As expected, the effects of calpain were Ca^{2+} -dependent as follows: there was no VGAT cleavage in a Ca^{2+} -free medium (containing 1 mM EGTA), but tVGAT formation was observed at physiological free calcium concentrations (100 nM and higher). As expected, VGAT cleavage in a Ca^{2+} -containing medium was sensitive to the calpain inhibitor MDL28170 (50 μM). Synaptic vesicle extracts typically showed two bands immunoreactive with the anti-VGAT antibody. The most abundant band, corresponding to an apparent molecular mass of 58 kDa, is analogous to the transporter identified in cultured neurons. The second band, with an apparent molecular mass of 50–52 kDa, may correspond to an isoform of the vesicular GABA transporter that has been described previously (Chessler et al., 2002; Ebihara et al., 2003; Oh et al., 2005; Suckow et al., 2006). In the experiments with cultured hippocampal neurons, this putative isoform was only observed with some batches of the polyclonal antibody, and it was less abundant than the 58 kDa band (data not shown).

The role of calpains in VGAT cleavage under excitotoxic conditions was further investigated *in vivo*, using the intrahippocam-

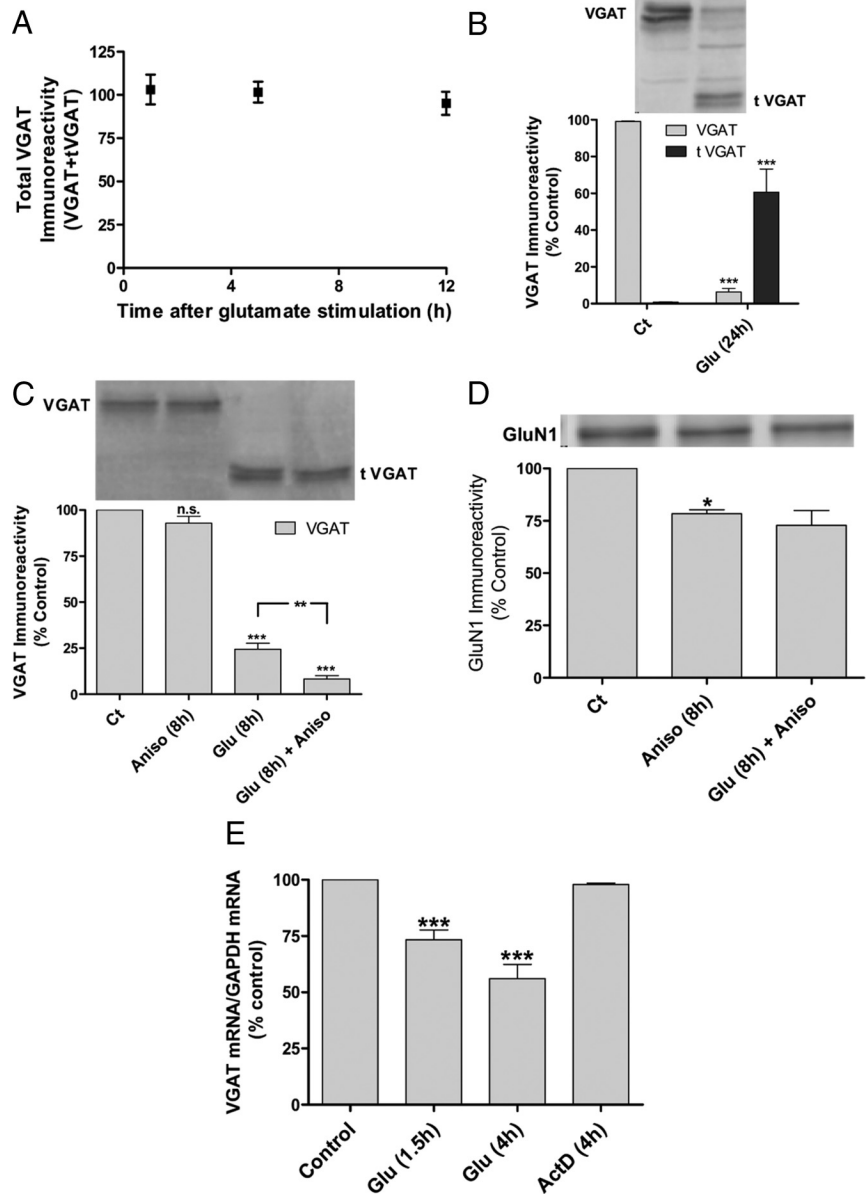


Figure 4. tVGAT has a long half-life. **A–C**, Cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate (125 μM glutamate, 20 min) and further incubated in culture-conditioned medium for the indicated period of time. VGAT and tVGAT protein levels were determined by Western blot. The results are the average \pm SEM of 4 (**A**, **B**, **D**, **E**) or 4–12 (**C**) independent experiments, performed in different preparations (in **A** the results were taken from Fig. 1A). Where indicated the cells were stimulated in the presence or in the absence of the protein synthesis inhibitor anisomycin (5 μM), and were further incubated in culture-conditioned medium for 8 h, in the presence or in the absence of the translation inhibitor. **E**, Cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate (125 μM glutamate, 20 min) and further incubated in culture-conditioned medium for the indicated periods of time. Alternatively, cultures were incubated with the transcription inhibitor actinomycin D (1.5 μM) for 4 h. Total RNA was extracted and VGAT and *Gapdh* mRNA were semiquantified through real-time PCR. Statistical analysis was performed using one-way ANOVA followed by Dunnett's (**A**) or Bonferroni's (**B–E**) multiple comparison tests. * $p < 0.05$, *** $p < 0.001$, compared with the control; *** $p < 0.01$ for the indicated comparison; n.s., not significant.

pal kainate injection and transgenic mice overexpressing calpastatin (h/CAST), a natural inhibitor of calpains (Higuchi et al., 2005; Takano et al., 2005). The animals were genotyped for the presence of the additional calpastatin human gene introduced in the mice genome, to ensure that all mice used in the experiments were transgenic, overexpressing calpastatin. Kainate injection induced VGAT cleavage in the hippocampus of wild-type mice but not in transgenic h/CAST mice, which display low calpain activity (Fig. 6A). As expected, no changes were observed in

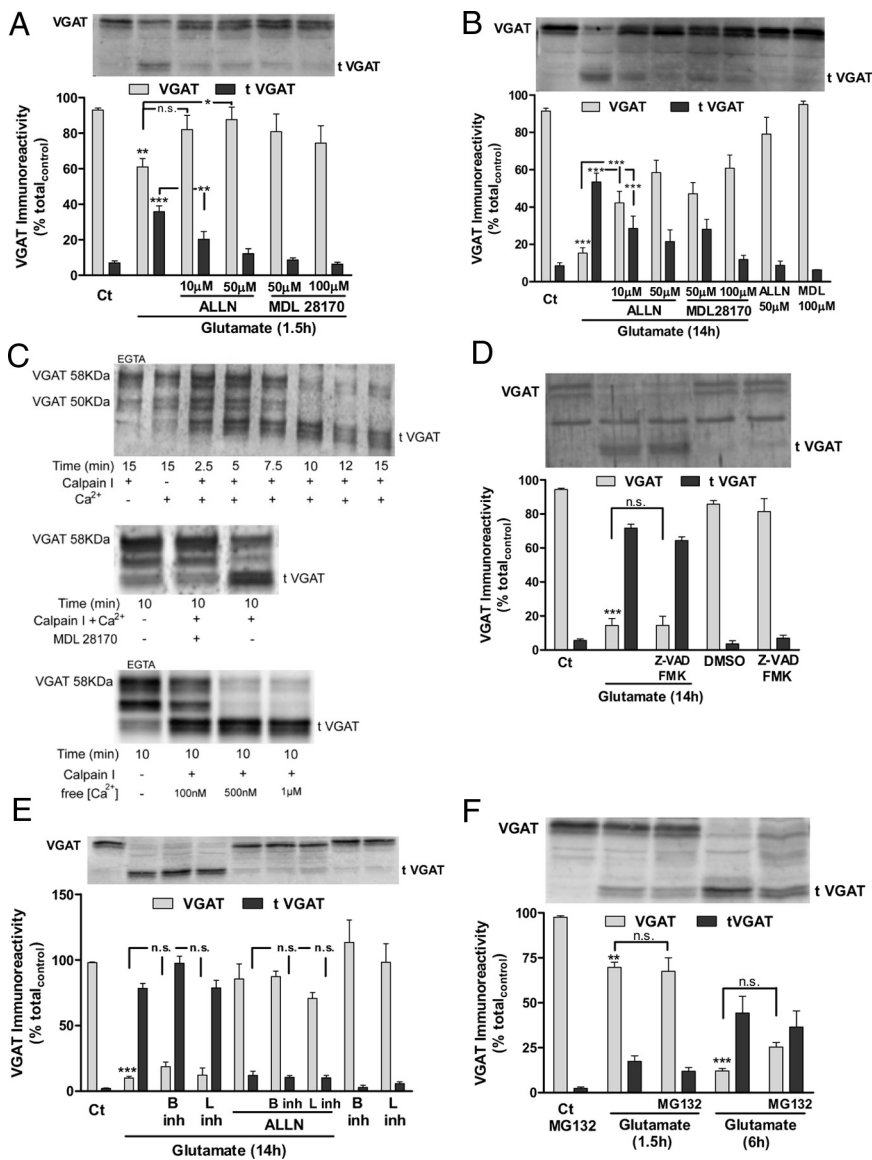


Figure 5. Calpain cleaves VGAT giving rise to tVGAT. **A, B,** Cultured hippocampal neurons were pre-incubated (2 h) with the calpain inhibitors MDL 28170 (50 and 100 μM) or ALLN (10 and 50 μM), before and during glutamate stimulation (125 μM, 20 min). The cells were then incubated in culture-conditioned medium for 1.5 h (**A**) or 14 h (**B**) with calpain inhibitors. The results are the average ± SEM of at least five different experiments performed in independent preparations. **C,** Cerebrocortical synaptic vesicles (5 μg) were incubated with recombinant calpain I (2.5U/ml) for the indicated periods of time at 37°C, in the presence (100 nM, 500 nM, 1 μM, 2.5 mM Ca²⁺) or in the absence (1 mM EGTA) of calcium. Where indicated the effect of the calpain inhibitor MDL 28170 (50 μM) was tested. The results are representative of at least three independent experiments. **D,** The effect of the caspase inhibitor Z-VAD-FMK (50 μM) (and DMSO as vehicle) on glutamate-evoked VGAT cleavage was tested using the experimental conditions described in **B**. The effect of cathepsin B (Ca-074 Me, 50 μM) and L (Inhibitor III, 10 μM) inhibitors is shown in **E**. Cumulative inhibition of calpains (ALLN, 50 μM) and cathepsins was also tested in **E**. **F** shows experiments where the effect of the proteasome inhibitor MG132 (1 μM) on VGAT cleavage was tested. In the latter experiments the cells were preincubated for 30 min with the proteasome inhibitor before the glutamate stimulation. After excitotoxic stimulation, the cells were incubated in culture-conditioned medium in the presence or in the absence of the tested inhibitor, for the indicated periods of time. In all experiments the extracts were analyzed by Western blot with an antibody against VGAT. In the results of **D–F** the average ± SEM of at least four experiments was performed in independent preparations. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001; n.s., not significant, compared with the control protein levels for VGAT or tVGAT or for the indicated comparisons.

the contralateral hemisphere. Control experiments, consisting in the injection of PBS, the kainate vehicle, into the hippocampus, did not induce VGAT cleavage in WT or transgenic mice. These results show that calpain also plays a role in VGAT cleavage under excitotoxic conditions *in vivo*. Kainate toxicity under the experimental conditions used was confirmed by staining brain slices

with cresyl violet. In contrast with the increase in cell death observed upon injection of kainate in WT mice, no significant effects were observed in transgenic mice overexpressing calpastatin, showing that calpain contributes to neuronal death (Fig. 6B).

VGAT is truncated in two cleavage sites, in the N-terminal region

According to the predicted topology, the VGAT N-terminal region is directed toward the cytoplasm, whereas the C-terminal region is located within the synaptic vesicles (McIntire et al., 1997; Martens et al., 2008). This suggests that the N-terminal region of the transporter may be targeted by calpains under excitotoxic conditions. This hypothesis is further supported by the presence of a putative PEST sequence in the N-terminal region of the transporter (PEST score: +11.17). These amino acid sequences, which are enriched in proline, glutamate, serine, and threonine, are typically targeted by calpains that may then cleave neighbor sequences (Rogers et al., 1986; Rechsteiner and Rogers, 1996) (Fig. 7A). As a first approach to further investigate the VGAT amino acid sequence(s) targeted by calpains, we performed Western blot experiments using antibodies that bind specifically to different epitopes on VGAT, in the N- and C-terminal regions of the protein. Truncated VGAT reacted with the antibodies raised against the C- (amino acids 500–525) and N- (amino acids 75–87) terminal regions of VGAT, but was not detected with an antibody against amino acids 1–20, suggesting that the cleavage site is within the N-terminal region of VGAT, probably between amino acids 20–75 (Fig. 7A,B).

To further evaluate the putative cleavage site(s) in the N-terminal region of VGAT we used the algorithm of Tompa et al. (2004), which allows predicting amino acid sequences targeted by calpain. The results of Figure 7C show that the PEST sequence (amino acids 64–83) is located near the region where the highest scores are found (Fig. 7C). Since it was proposed that calpains cleave their substrates in rather disordered segments of the protein (Tompa et al., 2004), we used bioinformatics tools to predict the disorder tendency along the VGAT amino acid sequence (Ishida and Kinoshita, 2008). The results of Figure 7D show that the protein is more disordered at the N-terminus,

particularly in the sequence between amino acids 50–115.

A careful analysis of the results of the Western blot experiments using extracts from cultured hippocampal neurons subjected to excitotoxic conditions and cerebrocortical vesicles incubated with calpain shows two close immunoreactive bands, both for the full-length and the truncated VGAT. The double

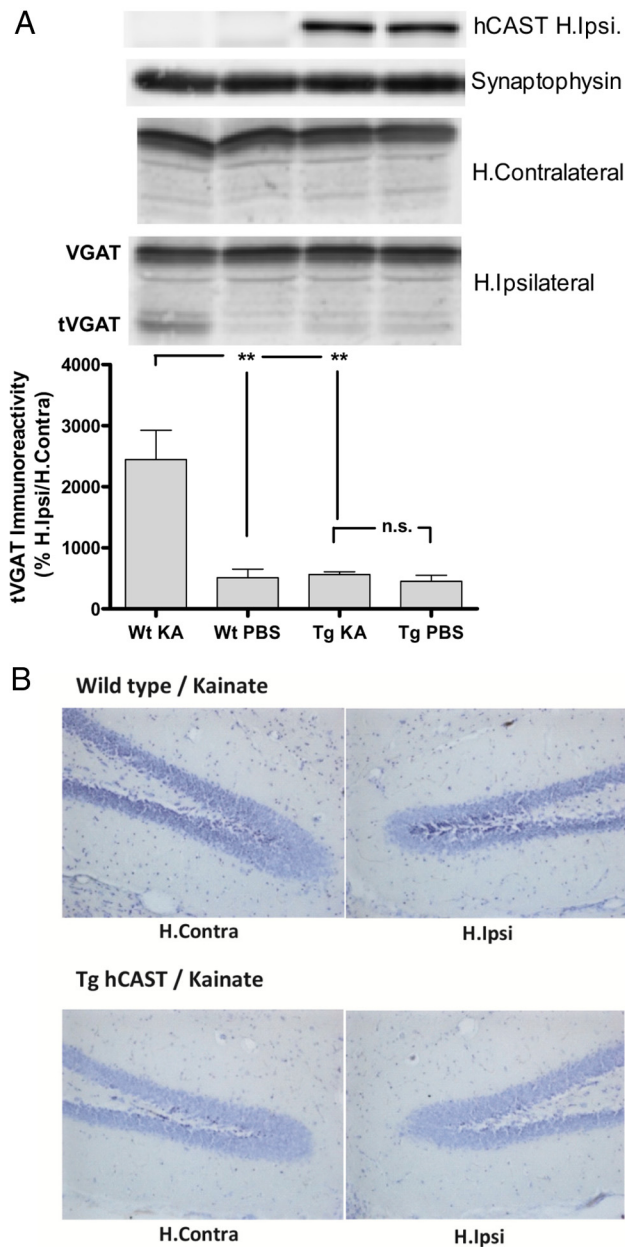


Figure 6. Kainate-induced VGAT cleavage is abrogated in hCAST-Tg mice. **A**, Adult Wt and Tg h/CAST mice (C56BL6) were injected with kainate (0.1 nmol in 0.3 μ l of phosphate buffer) in the right hippocampus, and 24 h later the ipsilateral and the contralateral hippocampi were collected from the coronal sliced brain. Hippocampal extracts were made and analyzed by Western blot against VGAT, human calpastatin, and synaptophysin (as loading control). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test (** $p < 0.01$; n.s., not significant). **B**, Adult Wt and Tg h/CAST mice were injected with kainate as in **A** and 24 h later the animals were perfused with phosphate buffer and paraformaldehyde. The brain was collected and sliced (30 μ m). Slices were stained with cresyl violet. Images show staining of the ipsilateral and contralateral hippocampal dentate gyrus. The results are representative of at least three independent experiments, performed in different animals.

band for full-length VGAT has already been described as a phosphorylated and dephosphorylated forms of VGAT, although it remains to be determined how phosphorylation affects the activity of the transporter (Bedet et al., 2000). Accordingly, in extracts incubated with CIP VGAT was observed as a single band. However, CIP showed no effect on the mobility of two close bands corresponding to truncated VGAT, suggesting that excitotoxic stimulation gives rise to two different cleavage products (Fig. 7E).

The calpain cleavage sites were determined by incubating recombinant calpain with a GST-fusion protein with the N-terminal region of VGAT. The cleavage products were separated by SDS-PAGE, transferred to a PVDF membrane, and sequenced by N-terminal Edman sequencing. Three cleavage sites were observed, at amino acids 7, 52, and 60 (Fig. 7F). However, only two of these sites (amino acids 52 and 60) matched the results obtained in neuronal cultures. The GST protein alone was not affected by calpain (Fig. 7F). To confirm the VGAT cleavage site(s) following excitotoxic stimulation, PC12 cells were transfected with a construct corresponding to VGAT truncated at amino acids 52 or 60 in the pcDNA3.1VGAT plasmid vector. Western blot experiments were performed to compare the mobility of the truncated transporter formed upon excitotoxic stimulation of hippocampal neurons and the transporters expressed in transfected PC12 cells. The results show a similar mobility in SDS-PAGE of tVGAT expressed in hippocampal neurons and the truncated VGAT expressed in PC12 cells (Fig. 7G).

VGAT cleavage changes the distribution of the protein along axons

Since the N-terminal region of VGAT is located outside the synaptic vesicles, it is likely to play a role in the trafficking of the protein along the cell body and neurites, through interaction with other cytoplasmic proteins. To further investigate how VGAT cleavage affects the subcellular distribution of the protein, we compared the distribution of GFP fusion proteins with VGAT or tVGAT. We used the plasmid vector pEGFP-N1 that fuses GFP to the C-terminus of VGAT, and the truncated form of GFP-VGAT was produced by *in situ* mutagenesis. Control immunocytochemistry experiments showed that the distribution of GFP-VGAT colocalized with endogenous VGAT along neurites in cultured hippocampal neurons (data not shown), showing that the fusion of GFP with the C-terminus of VGAT did not alter its intracellular traffic.

The cellular distribution of GFP-VGAT and GFP-tVGAT (1–52 and 1–60) was compared in cultured striatal neurons instead of cultured hippocampal neurons because the former preparation is enriched in GABAergic neurons. The cells were cultured for 11 d to allow synaptic differentiation, and the distribution of the GFP fusion proteins was analyzed by confocal microscopy in GABAergic neurons, identified by immunocytochemistry with an antibody against GABA. GFP-VGAT showed a punctate distribution along neurites, which colocalized with synaptophysin, a common synaptic marker (Fig. 8A). In contrast, the GFP-tVGAT forms [GFP-VGAT Δ (1–52) (Fig. 8B) and GFP-VGAT Δ (1–60) (Fig. 8C)] were distributed homogeneously along neurites with no colocalization with synaptophysin. Control Western blot experiments showed that the fusion proteins expressed in PC12 cells had the expected apparent molecular weight and were recognized by both VGAT and GFP antibodies (Fig. 8D).

The distribution of endogenous VGAT was also compared in cultured hippocampal neurons under control conditions and after excitotoxic stimulation with glutamate (125 μ M, 20 min). Immunocytochemistry experiments with an antibody against the N-terminal region of VGAT (amino acids 75–87) show a significant reduction in the punctated distribution of VGAT along neurites 1.5 and 8 h after the toxic insult (Fig. 8E). At the latter time point >80% of VGAT was in the truncated form (see Fig. 1A), showing a homogeneous distribution along neurites. This pattern of VGAT distribution resembles the immunoreactivity observed for the transfected truncated proteins (Fig. 8B,C). Together, the results clearly show that the cleavage of VGAT alters the subcel-

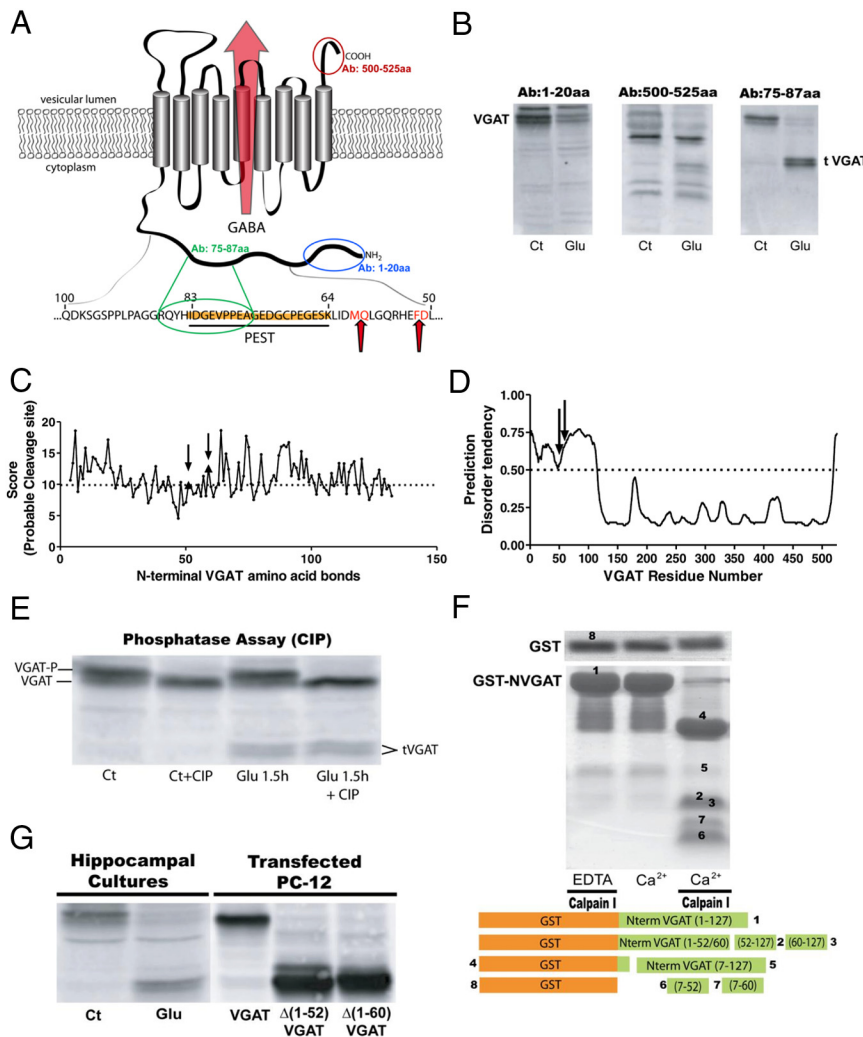


Figure 7. VGAT is truncated in two cleavage sites in the N-terminal region. **A**, Predicted topology of VGAT, showing the antibody (Ab) binding sites and the calpain cleavage sites identified (51–52 and 59–60). The antibody against amino acids 75–87 recognizes both the full-length and truncated VGAT (Fig. 1A). **B**, Western blot of extracts prepared from control and glutamate (125 μ M, 20 min) stimulated cultured hippocampal neurons. The extracts were prepared 12 h after the excitotoxic insult, and the membranes were probed with antibodies against the indicated VGAT amino acid sequences. **C**, Analysis of the probability of cleavage by calpain in the N-terminal region of VGAT based on the model for prediction of calpain cleavage sites published in the study by Tompa et al. (2004). Arrows point to the predicted calpain cleavage sites identified in the present work. **D**, Prediction of the disorder tendency of VGAT based on a bioinformatic analysis of the amino acid sequence of the protein (Ishida and Kinoshita, 2008). Arrows point to the predicted calpain cleavage sites identified in the present work. **E**, Extracts from cultured hippocampal neurons subjected to excitotoxic stimulation with glutamate (and further incubated in culture-conditioned medium for 1.5 h) were incubated with CIP (2U/ μ l) for 1 h at 37°C, in the presence of protease inhibitors. After incubation, the extracts were analyzed by Western blot with an antibody against VGAT. **F**, The GST–N-terminal VGAT fusion protein and GST protein (100 μ g) were incubated with or without recombinant calpain I (2.5U/ml) in the presence of calcium (2.5 mM Ca^{2+}), for 15 min at 37°C. The peptides were separated by SDS-PAGE and were stained immediately (or after transfer to a PVDF membrane) with Coomassie blue. The PVDF membrane with the indicated fragments was processed for N-terminal Edman sequencing. **G**, Comparison of VGAT and tVGAT expressed in cultured hippocampal neurons subjected to excitotoxic stimulation with glutamate and in transfected PC12 cell lines expressing the cDNA of VGAT (pcDNA3.1-VGAT) and the truncated VGAT forms created by mutagenesis [pcDNA3.1-VGAT Δ (1–52) and pcDNA3.1-VGAT Δ (1–60)]. Extracts were analyzed by Western blot with an antibody against VGAT.

lular distribution of the protein, and this may affect GABAergic synaptic transmission because tVGAT is no longer at the synapses.

Proposed mechanism of VGAT cleavage and tVGAT formation in the GABAergic synapses

A few intrinsic signals that control the trafficking of vesicular neurotransmitter transporters have been identified, including the dileucine motif present in multiple transporters, an acidic cluster, present in the vesicular monoamine transporter 2

(VMAT2), and a polyproline motif characterized in the vesicular glutamate transporter 1 (VGLUT) (Fei et al., 2008). However, up to now no such signals have been identified for VGAT. Analysis of the N-terminal region of VGAT shows four putative trafficking signals (Fig. 9A), as follows: two dileucine motifs (A, C), which can be composed of two leucines or one leucine and an isoleucine, one acidic motif (B) and a proline motif (D). When VGAT is truncated by calpain the first two motifs (A, B) are lost, but the others (C, D) are kept in the truncated proteins and may affect the subcellular distribution of the transporter. To further investigate how VGAT cleavage affects its subcellular distribution, we generated by directed *in situ* mutagenesis a mutated form of GFP–VGAT lacking amino acids 40–90 [GFP–VGAT Δ (40–90)]. This form of VGAT does not contain the PEST sequence and the identified calpain cleavage sites, but is endowed with three of the four putative trafficking signals discussed above. Hippocampal neurons transfected with GFP–VGAT Δ (40–90) were subjected to excitotoxic stimulation with glutamate (125 μ M, 20 min), and the amount of mutant transporter present in the cells was determined by Western blot, with an antibody against GFP. The results show that the mutant transporter is resistant to calpain cleavage in hippocampal neurons stimulated under conditions that lead to the cleavage of the full-length transporter (Fig. 9B). Immunocytochemistry experiments were performed to determine the cellular distribution of GFP–VGAT Δ (40–90) in transfected cultured GABAergic hippocampal neurons. GABAergic neurons were identified using an antibody against GABA and the results show a punctate distribution of VGAT along neurites.

Discussion

In this work, we show that VGAT is cleaved under excitotoxic conditions *in vitro* and *in vivo*, and in transient focal cerebral ischemia, giving rise to a truncated product that lacks a punctate synaptic distribution. VGAT cleavage was mediated by calpains, and the stability of the cleavage product suggests that the protease plays a modulatory role rather than a destructive effect in excitotoxic neuronal damage. These findings are in agreement with the key role played by calpains in excitotoxic neuronal damage and in brain ischemia (Bever and Neumar, 2008).

Several pieces of evidence indicate that calpains mediate VGAT cleavage under excitotoxic conditions, as follows: (1) chemical inhibitors of calpain prevented the formation of a VGAT cleavage product in cultured neurons subjected to excitotoxic stimulation with glutamate; (2) transgenic mice overexpressing calpastatin, the

endogenous calpain inhibitor, showed a decrease in kainate-evoked VGAT cleavage; (3) incubation of synaptic vesicles with recombinant calpain in the presence of physiological calcium concentrations induces the cleavage of VGAT. Calpains are also activated in the striatum and in the cerebral cortex after MCAO, contributing to neuronal death (Takagaki et al., 1997; Bevers and Neumar, 2008), and may mediate the observed cleavage of VGAT in mice exposed to this model of transient focal brain ischemia. VGAT cleavage in the hippocampus of rats following systemic injection of kainate may also arise from calpain activation. In fact, activation of calpains was shown to contribute to neurodegeneration in the hippocampus in this model of temporal lobe epilepsy (Goodman, 1998; Araújo et al., 2008). Interestingly, 24 h after MCAO there was a recovery of full-length VGAT protein levels, suggesting that the functional consequences of the cleavage of the transporter may be more relevant in the first hours after ischemia, when excitotoxicity takes place, and alterations in GABAergic synaptic transmission may interfere in the balance excitation/inhibition. VGAT cleavage was also observed under conditions that were previously shown to increase the excitatory activity in cultures of hippocampal neurons, known as chemical LTP, and this effect was also abrogated in the presence of the calpain inhibitor ALLN. The cleavage of VGAT under these conditions was not as significant as under excitotoxic conditions (compare Figs. 1A, 3), most likely due to a controlled activation of calpains. Accordingly, activation of extrasynaptic NMDA receptors was shown to be more efficient in inducing the cleavage of calpain substrates than stimulation of synaptic receptors (Xu et al., 2009). However, the calpain-mediated cleavage of VGAT under conditions leading to long-term changes in synaptic activity correlates with the reported role of calpain in the regulation of synaptic function and memory formation under normal physiological conditions (Liu et al., 2008).

The total VGAT protein levels (full-length + cleaved transporter) in hippocampal neurons subjected to excitotoxic conditions was not changed 12 h after the insult, suggesting that the cleavage of the transporter represents a posttranslational modification. The truncated form of the transporter was highly stable, being present in cultured hippocampal neurons 24 h after the excitotoxic insult. In addition to VGAT, calpains cleave other proteins in brain ischemia and under excitotoxic conditions, giving rise to stable cleavage products (Chan and Mattson, 1999; Hou et al., 2006; Cao et al., 2007; Xu et al., 2007; Bevers and Neumar, 2008; Gascón et al., 2008). Excitotoxic stimulation also decreased the mRNA for VGAT, which may prevent *de novo* synthesis of the transporter to compensate for the loss of the full-length protein in surviving neurons. This loss of VGAT mRNA

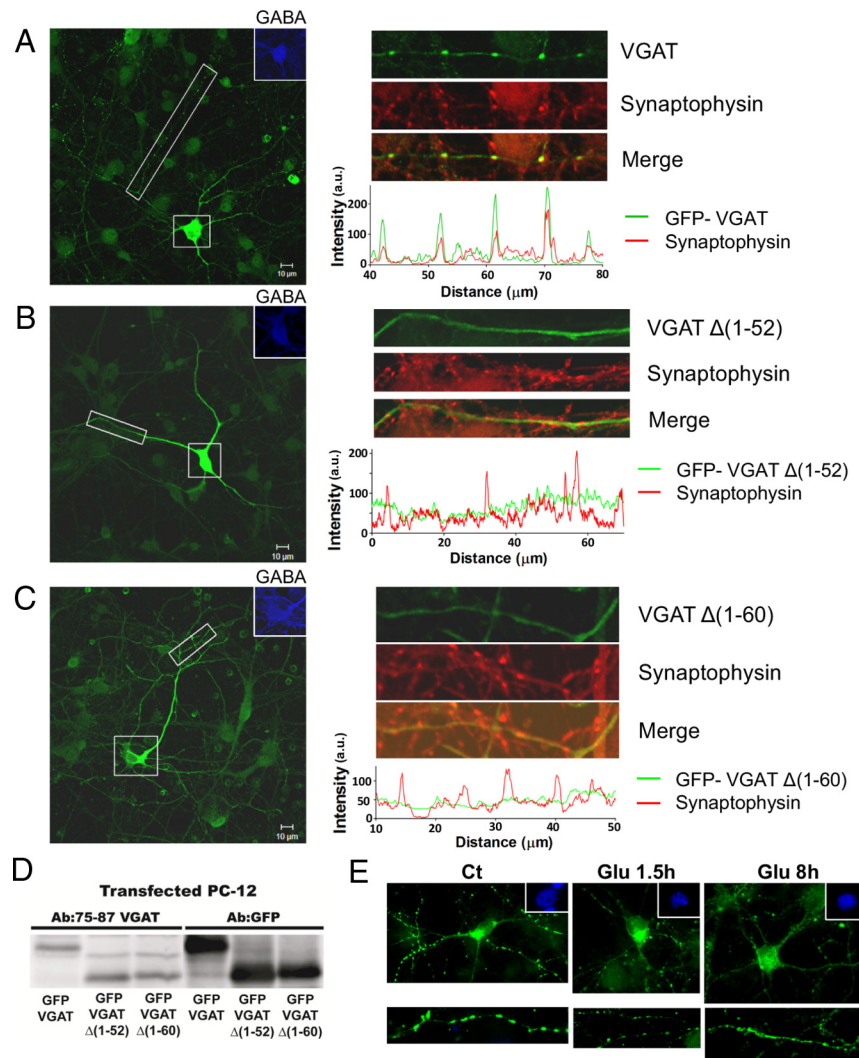


Figure 8. VGAT cleavage changes the distribution of the protein along axons. **A**, Cultured striatal neurons (11 DIV) were transfected with GFP-VGAT (**A**), GFP-VGAT Δ (1–52) (**B**), or GFP-VGAT Δ (1–60) (**C**). Fixed and permeabilized cells were incubated with primary antibodies against GFP (green), synaptophysin (red), and GABA (blue). Transfected GABAergic neurons (stained with the antibody against GABA) are shown on the left (**A–C**) and zoom images of their neurites on the right. The pixel intensities for VGAT and synaptophysin labeling along axons is shown in the graphs. **D**, GFP-VGAT and GFP fusion protein with truncated forms of VGAT [GFP-VGAT Δ (1–52), GFP-VGAT Δ (1–60)] were transfected in PC-12 cells, and cellular extracts were analyzed by Western blot using an antibody against VGAT amino acids 75–87 or against GFP. **E**, Cultured hippocampal neurons with 7 DIV were subjected to excitotoxic stimulation with glutamate (125 μ M, 20 min) and further incubated in culture-conditioned medium for 1.5 or 8 h. After fixation and permeabilization, the cells were incubated with a primary antibody that reacts with the N-terminal region of VGAT (amino acids 75–87) and the nuclear marker Hoechst 33342 (0.5 μ g/ml). VGAT detection was performed with a secondary antibody labeled with Alexa 488.

is likely due to active degradation rather than inhibition of transcription, since transcription inhibition for the same period of time was without effect. A decrease in VGAT mRNA and downregulation of VGAT protein levels was also observed in the cerebral cortex of rats subjected to transient focal cerebral ischemia (Raghavendra Rao et al., 2002; Vemuganti, 2005).

The cleavage of VGAT under excitotoxic conditions was biphasic, consisting in an early calpain-sensitive step followed by a delayed phase ($t = 3–8$ h), with a lower sensitivity to calpain inhibitors. The first step may be mediated by the μ -calpain isoform, which is activated by calcium at micromolar concentrations. The late VGAT cleavage may be mediated by m-calpains, which require higher calcium concentrations, in the millimolar range (Goll et al., 2003).

Western blot experiments using antibodies against the N- and C-terminal regions of VGAT showed that the transporter is

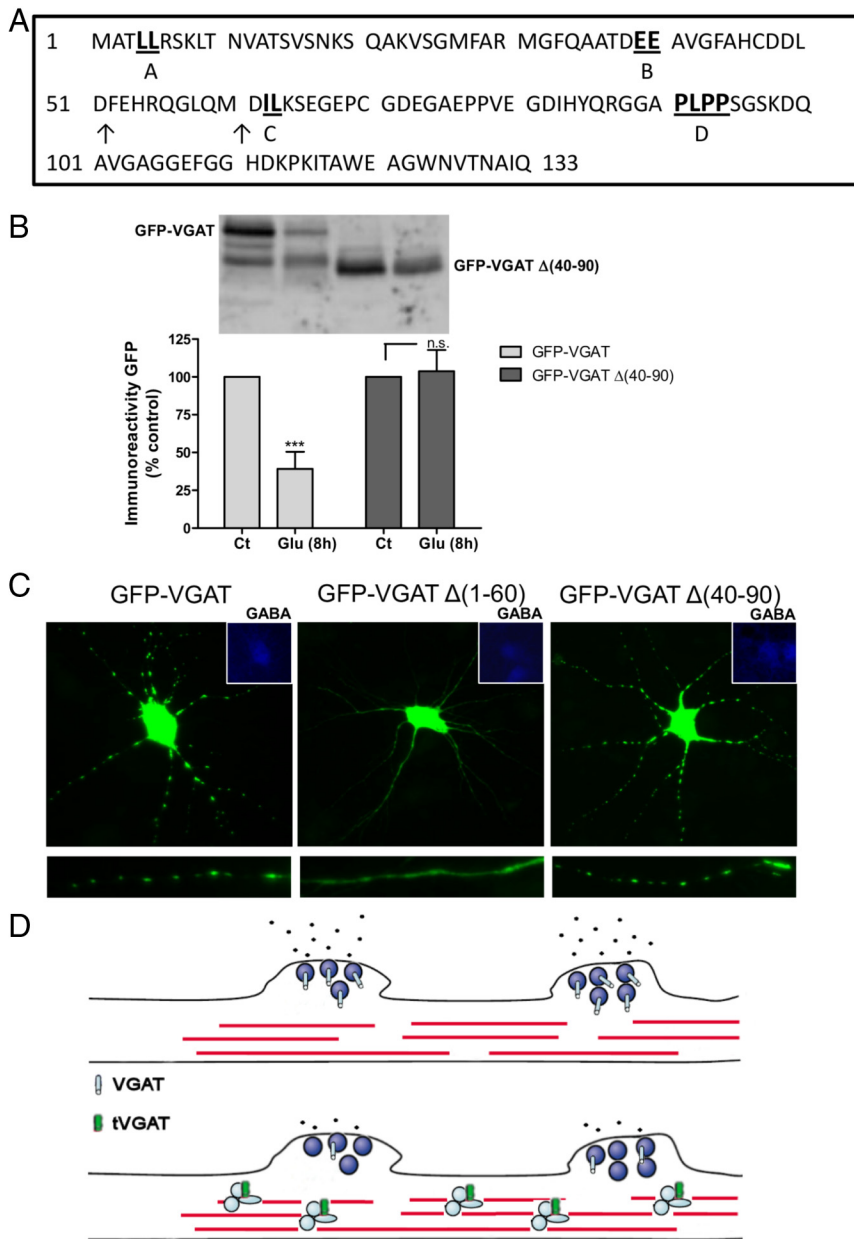


Figure 9. Proposed mechanism of VGAT cleavage and tVGAT formation in the GABAergic synapses. **A**, Cytoplasmic N-terminal region of VGAT is shown with putative trafficking signals underlined (**A–D**), identified based on the characterization reported for other vesicular transporters. The arrows indicate the calpain cleavage sites described in this work. **B**, Cultured hippocampal neurons (7 DIV) transfected with GFP-VGAT or a mutated form of GFP-VGAT lacking amino acids 40–90 (Δ 40–90; the deleted sequence contains the calpain cleavage sites and a PEST sequence) were challenged with glutamate (125 μ M, 20 min) and further incubated in culture-conditioned medium for 8 h. Excitotoxic stimulation was conducted 72 h after transfection and the extracts were analyzed by Western blot, using an antibody against GFP. Statistical analysis was performed using the one-way ANOVA followed by Bonferroni’s multiple comparison test (***) $p < 0.001$; n.s., not significant). **C**, Cultured hippocampal neurons (10 DIV) were transfected with GFP-VGAT, GFP-VGAT Δ (1–60), and GFP-VGAT Δ (40–90), and 3–4 d later the cells were fixed and permeabilized for immunocytochemistry. The cells were incubated with primary antibodies against GFP (green) and GABA (blue). The figure shows transfected GABAergic neurons (stained with the antibody against GABA) expressing the GFP-VGAT forms. The zoom images show the distribution of the transporter along axons and the GABA stained soma (inset). **D**, Diagram showing the effect of VGAT cleavage on the distribution of the protein along axons. VGAT cleavage at the N-terminal region by calpain may eliminate trafficking signals that determine the subcellular distribution of the protein. Therefore, tVGAT is not accumulated in the vesicles present in synaptic regions. This is likely to affect the amount of GABA stored in synaptic vesicles accumulated at the synapse and may decrease the synaptic release of the neurotransmitter.

cleaved by calpain at the N-terminal region (Fig. 7B). According to the predicted topology (Fig. 7A), this region is directed toward the cytoplasm, and incubation of calpain with a recombinant GST fusion protein with the N-terminus of VGAT showed two

cleavage sites, at amino acids 52 and 60. This is further supported by the results showing that the truncated VGAT present in hippocampal neurons subjected to excitotoxic stimulation and the truncated VGAT [VGAT Δ (1–52) and (1–60)] expressed in PC12 cells display a similar mobility in SDS-PAGE (Fig. 7G). Analysis of the amino acid sequence of with the N-terminal region of VGAT shows a putative PEST sequence. These sequences are thought to constitute a signal for rapid intracellular proteolysis (Rogers et al., 1986; Rechsteiner and Rogers, 1996) and have been linked to protein cleavage by calpains (Shumway et al., 1999). However, whether PEST sequences provide a signal for cleavage by calpains is still an open issue because mutations of this sequence did not abolish calpain cleavage in some proteins and there are calpain substrates that lack PEST sequences (Carillo et al., 1996). To further evaluate the putative cleavage site(s) in the N-terminal region of VGAT, we used the algorithm of Tompa et al. (2004), which allows predicting amino acid sequences targeted by calpain (Fig. 7C). The sequences near amino acids 52 and 60 in the N-terminal region of VGAT show high scores, characteristic of calpain cleavage sites. Interestingly, the N-terminal region of VGAT is one of the most disordered segments of the protein, and different studies have proposed that calpains cleave their substrates in rather disordered regions (Fontana et al., 1997; Tompa et al., 2004).

The studies using GFP fusion proteins with the full-length VGAT and the truncated transporters (at amino acids 52 and 60) showed clear differences in the distribution of the transporters along axons. In contrast with the full-length protein, which showed a typical synaptic punctate distribution, the truncated proteins (lacking amino acids 2–51 or 2–59) were found along neurites with no colocalization with synaptophysin. Cultured hippocampal neurons subjected to excitotoxic stimulation with glutamate also showed a significant decrease in the punctate distribution of the endogenous VGAT, similarly to the distribution of the truncated transporters. These results show that cleavage of VGAT affects the targeting of the transporter to the synapse and/or its retention at the nerve terminals, which is likely to have an impact on the exocytotic release of GABA. The changes in the distribution of VGAT

upon cleavage at the N-terminal region resemble those observed in *unc-46 Caenorhabditis elegans* mutants (Schuske et al., 2007). UNC-46 is a homolog protein of LAMP (lysosomal associated membrane protein) and is localized at synapses, where it acts as a

sorting factor that targets vesicular GABA transporters to synaptic vesicles. In the *unc-46* mutants the vesicular GABA transporters are diffusely spread along the axon and the mislocalization of the transporters significantly reduces the frequency of GABA currents (Schuske et al., 2007). Based on these findings it is tempting to suggest that VGAT cleavage at the N-terminal region may prevent the interaction with binding partners responsible for targeting the transporters to the synapse, thereby affecting exocytotic GABA release.

The N-terminal region of VGAT contains several amino acid sequences that may play a role in synaptic delivery, which can be predicted based on studies performed for other vesicular neurotransmitter transporters (Fig. 9A). To further address this issue we investigated the cellular distribution of a truncated form of VGAT lacking amino acids 40–90 [GFP-VGAT Δ (40–90)], which does not contain the PEST sequence and the identified calpain cleavage sites, but possesses three of the four putative trafficking signals. This truncated transporter was not cleaved in hippocampal neurons subjected to excitotoxic conditions but showed a typical synaptic punctate distribution. These results are in agreement with the calpain cleavage sites identified based on the studies using recombinant calpain and the GST fusion protein with the N-terminal region of VGAT, and suggest that the PEST sequence may be important in the cleavage of the transporter. Furthermore, they suggest that the LL and EE motifs found in the N-terminal region of VGAT are important in the synaptic delivery of the protein.

Together, the results indicate that calpain-mediated VGAT cleavage under excitotoxic conditions gives rise to a truncated form of the transporter lacking sequences responsible for synaptic targeting (Fig. 9D). A similar effect may occur, although to a lower extent, in synapses undergoing high-frequency stimulation. VGAT cleavage by calpain leads to the formation of a new and stable VGAT form, tVGAT, which is not targeted to the synapse. This is expected to decrease the release of GABA by exocytosis, thereby changing the equilibrium between excitatory and inhibitory synaptic activity in the early phases after the excitotoxic insult.

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