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GABARAPL1 (GEC1) antibodies
Target one protein, get one free!

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Atg8 is a yeast protein involved in the autophagic process and in particular in the elongation of autophagosomes. In mammals, several orthologs have been identified and are classed into two subfamilies: the LC3 subfamily and the GABARAP subfamily, referred to simply as the LC3 or GABARAP families. GABARAPL1 (GABARAP-like protein 1), one of the proteins belonging to the GABARAP ($GABA_A$ receptor-associated protein) family, is highly expressed in the central nervous system and implicated in processes such as receptor and vesicle transport as well as autophagy. The proteins that make up the GABARAP family demonstrate conservation of their amino acid sequences and protein structures. In humans, GABARAPL1 shares 86% identity with GABARAP and 61% with GABARAPL2 (GATE-16). The identification of the individual proteins is thus very limited when working in vivo due to a lack of unique peptide sequences from which specific antibodies can be developed. Actually, and to our knowledge, there are no available antibodies on the market that are entirely specific to GABARAPL1 and the same may be true of the anti-GABARAP antibodies. In this study, we sought to examine the specificity of three antibodies targeted against different peptide sequences within GABARAPL1: CHEM-CENT (an antibody raised against a short peptide sequence within the center of the protein), PTG-NTER (an antibody raised against the N-terminus of the protein) and PTG-FL (an antibody raised against the full-length protein). The results described in this article demonstrate the importance of testing antibody specificity under the conditions for which it will be used experimentally, a caution that should be taken when studying the expression of the GABARAP family proteins.

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Introduction

Gabarapl1/gec1 (*gabarap-like 1 protein/glandular epithelial cell 1*) is an estrogen-regulated gene that belongs to the *gabarap ($GABA_A$ receptor-associated protein)* family of genes. It has been demonstrated that GABARAPL1 acts in receptor and vesicle transport thanks to its interaction with tubulin and the $GABA_A$ and kappa-opioid receptors.[1,2] GABARAPL1 also plays a role, like GABARAP, GATE-16 and LC3, in the process of autophagy and can be now considered as an autophagic marker as well.[3] The exact role of GABARAPL1 in intracellular transport and autophagy, however, is still unanswered, and will require further studies to address the remaining uncertainties. As with any experiment, the proper tools are critical to its success. As such, one essential technical question will have to be answered before the in vivo role of GABARAPL1, and similarly, the role of its closest homolog, GABARAP, can be elucidated: the question of the specificity of the currently available polyclonal anti-GABARAPL1 and anti-GABARAP antibodies. Indeed, we know that these two proteins share 86% identity at the amino-acid level (**Fig. 1**) and that the crystal structures of these proteins present identical tri-dimensional structures, similar to that of the ubiquitin protein.[4-6] These shared features make it very difficult to produce a specific antibody directed against only one of these two proteins. Moreover, in humans, GABARAPL1 shows 61% identity with GATE-16,[7] 54% with yeast Atg8 (autophagy-related protein),[8] and 28.8% with MAP-LC3 (light chain 3 of microtubule associated protein).[9-11]

GABARAPL1 was first discovered in 1993 and described as part of the GABARAP family in 2001.[12] When performing a far from exhaustive search on PubMed, we counted about 31 publications citing this protein (using three different names: GEC1, GABARAPL1 or GABARAP-L1). Within these 31 publications, only nine presented antibody-detection of the GABARAPL1 protein. The majority of these publications examine the expression of *gabarapl1* at the mRNA level.

Since the discovery of GABARAPL1 in our laboratory, we have endeavored to identify reliable tools to study this protein and have developed an appreciation for the technical problems linked to these tools. The main problem is that GABARAP and GABARAPL1 are so closely related (86% identity, **Fig. 1**) that their in vivo immunodistinction becomes very improbable. This problem can be easily circumvented when working with immunoblotting analysis. Although their calculated molecular weights are identical (14 kDa), GABARAPL1 displays a slower migration rate than GABARAP, allowing one to separate the two proteins with a

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GABARAPL1  MKFQYKEDHPFEYRKKEGEKIRKKYPDRVPVIVEKAPKARVPDLDKRKYLVPSDLTVGQF -60
GABARAP    MKFVYKEEHPFEKRRSEGEKIRKKYPDRVPVIVEKAPKARIGDLDKKKYLVPSDLTVGQF -60
              QYKEDHPFEYRKKEG                     KAPKARVPDLDKRKYLVPSDLTVGQF

GABARAPL1  YFLIRKRIHLRPEDALFFFVNNTIPPTSATMGQLYEDNHEEDYFLYVAYSDESVYGK -117
GABARAP    YFLIRKRIHLRAEDALFFFVNNVIPPTSATMGQLYQEHHEEDFFLYIAYSDESVYGL -117
           YFLIRKRIHLRPEDALFFFVNNTI
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Figure 1. Alignment of peptide sequences used to produce anti-GABARAPL1 antibodies with GABARAPL1 and GABARAP. The human amino-acid sequences (obtained from NCBI or GeneBank databases) of GABARAPL1 (NP_113600) and GABARAP (CAG47031) proteins were aligned using the NCBI Protein BLAST tool. The amino acid sequences of the peptides used to produce anti-GABARAPL1 are highlighted in blue (PTG-NTER, Proteintech, 18721-1-AP) and in green (CHEM-CENT, Chemicon, AB15278). Variation between the GABARAP and GABARAPL1 proteins are shown in red.

higher percentage of acrylamide in the gel and a longer migration time.[3,13] Immunohistological analysis of the endogenous proteins, however, does not provide an easy alternative to the necessity of specific antibodies. Indeed, many researchers have encountered this problem of cross-reactivity with both their 'homemade' and commercially bought antisera and purified antibodies raised against either GABARAPL1 or GABARAP.[1,2,14] To our knowledge, there are no available antibodies that can differentiate between these two proteins, explaining why so many laboratories have chosen to use the less problematic mRNA-based techniques to overcome this problem and study the expression of these genes in vivo. While these studies have allowed for a better characterization and understanding of *gabarapl1* gene expression and regulation, they do not explore GABARAPL1 protein expression and regulation.

In the past, GABARAPL1 has been the subject of only a handful of studies, while most laboratories focused their efforts on other members of the GABARAP and LC3 families such as GABARAP, GATE-16 and LC3 as well as the yeast homolog, Atg8. Our recent paper establishing GABARAPL1 as a new autophagic marker, however, may trigger a new wave of research on this protein. The interest in GABARAPL1 functionality, in particular in the brain where it is the most highly expressed gene of the family,[15] is likely to grow in the future. Before that time comes, we wish to present several pieces of data explaining our concerns about the detection of the GABARAPL1 protein in vitro and in vivo in regards to its high homology with the GABARAP protein.

Results

In this work, we studied three anti-GABARAPL1 antibodies among those that are commercially available: one directed against the full-length GABARAPL1 protein (PTG-FL, 11010-1-AP, Proteintech), a second raised against a short peptide sequence at the N-terminal of the protein (PTG-NTER, amino acids 4–18, QYK EDH PFE YRK KEG, 18721-1-AP, Proteintech) and a third raised against a longer peptide sequence in the center of the protein (CHEM-CENT, amino acids 35–84, KAP KAR VPD LDK RKY LVP SDL TVG QFY FLI RKR IHL RPE DAL FFF VNN TI, AB15278, Chemicon) (**Fig. 1**). All antibodies used are rabbit polyclonal antibodies. It is noteworthy that no monoclonal antibody directed against either GABARAPL1 or GABARAP is currently available.

To investigate the specificity of these antibodies against denatured GABARAPL1 and GABARAP, we performed protein gel blot analysis with HEK293 WT and FLAG-GABARAPL1-6HIS stably expressing HEK293 cell lysates (**Fig. 2A**). Antibodies directed against the FLAG peptide and Actin were used as internal controls of FLAG-GABARAPL1-6HIS protein expression and total amounts of loaded proteins, respectively. While these cell lines both express an easily detectable amount of GABARAP, endogenous GABARAPL1 expression is very weak, necessitating the use of the FLAG-GABARAPL1-6HIS stably transfected HEK293 cell line in order to more easily visualize GABARAPL1 in these lysates. All three antibodies tested displayed a relatively strong signal at approximately 18 kDa in size (**Fig. 2A**, lanes 1, 3 and 5), corresponding to FLAG-GABARAPL1-6HIS in the stably expressing cell line. In addition, the antibody raised against a central peptide of the GABARAPL1 protein (CHEM-CENT) revealed a second very intense band at approximately 15 kDa in size (**Fig. 2A**, lanes 5 and 6), corresponding to endogenous GABARAP. Surprisingly, despite the presence of a stably expressed recombinant GABARAPL1 protein this antibody produced a prominent band for the endogenous GABARAP protein, suggesting that this antibody equally recognizes the two proteins.

Given that protein-antibody interactions can vary depending on the amount of protein expressed, we chose to confirm these results with transient transfections of either GFP-GABARAPL1 or GFP-GABARAP prior to protein gel blot analysis of the protein lysates (**Fig. 2B**). An antibody directed against GFP was used as an internal control of the fusion proteins' expression in the cells. In accordance with the previous results, the antibody raised against the central peptide (CHEM-CENT) displayed a 40 kDa band for both GFP-GABARAPL1 and GFP-GABARAP (**Fig. 2B**, lanes 5 and 6). In contrast, when GABARAPL1 and GABARAP are overexpressed, only one antibody of the two previously identified to be specific remained so. The antibody produced with the N-terminal peptide (PTG-NTER) detected both the GFP-GABARAPL1 and the GFP-GABARAP (**Fig. 2B**, lanes 1 and 2), suggesting that this antibody is not specific when both proteins are overexpressed. The antibody directed against the full-length peptide (PTG-FL), however, continued to identify only the GABARAPL1 protein (**Fig. 2B**, lane 3), producing only one signal corresponding to the

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GFP-GABARAPL1. These results suggest that this antibody remains specific to GABARAPL1 whatever the levels of overexpressed proteins.

In order to examine the ability of these antibodies to discern between native GABARAPL1 and GABARAP, we performed liquid phase inhibition experiments followed by immunohistochemical analysis. Given the known high expression of *gabarapl1* mRNA in the mouse brain and the fact that GABARAPL1 intervenes in the transport of neuronal receptors, we chose to conduct these studies on mouse cerebral sections. The respective antibodies were incubated alone or with either full-length recombinant FLAG-GABARAPL1-6HIS or FLAG-GABARAP-6HIS for two hours prior to incubation on mouse coronal brain sections overnight followed by classical immunofluorescent detection methods. Staining intensity was then analyzed throughout the slice and representative images of three separate experiments were taken in the cortex. In the presence of the individual antibodies alone, the visible staining pattern varied from one antibody to another. The antibody raised against the full-length protein (PTG-FL) displayed the highest background and marked the cytoplasm of cells with a more intense vesicular staining within the cytoplasm as well as a fainter fiber-like staining pattern (**Fig. 3A**). The antibody raised against the N-terminal of the GABARAPL1 protein (PTG-NTER) displayed a similar staining pattern although the background staining was drastically reduced and fibril-like staining increased (**Fig. 3D**). Finally, the third antibody, raised against the central part of the GABARAPL1 protein (CHEM-CENT), displayed a much weaker signal in the cytoplasm of cells and a more prominent vesicular pattern throughout the section (**Fig. 3G**). After incubation with the FLAG-GABARAPL1-6HIS protein, all three antibodies were attenuated and little to no positive staining could be found in the corresponding sections, indicating that all three antibodies clearly recognize the GABARAPL1 protein in its native form (**Fig. 3B, E and H**). The results obtained from the incubation with the FLAG-GABARAP-6HIS protein, however, differed depending on the antibody in question. Incubation of the PTG-FL antibody with recombinant GABARAP did not appear to affect staining intensity at all (**Fig. 3C**), demonstrating that under the conditions used, this antibody is capable of distinguishing between GABARAPL1 and GABARAP. Incubation of the PTG-NTER antibody with recombinant GABARAP produced the exact opposite effect, with a complete attenuation of the visible signal on the corresponding tissue section (**Fig. 3F**). This was surprising since this antibody showed some specificity in protein gel blot experiments (**Fig. 2**). Finally, recombinant GABARAP, when incubated with the antibody produced by immunization with the central peptide (CHEM-CENT), reduced staining in the last experiment to about the same extent as incubation with recombinant GABARAPL1 (**Fig. 3I**). The two antibodies produced against peptide sequences within the GABARAPL1 protein were inhibited by incubation with recombinant GABARAP, suggesting that, under the conditions used, these antibodies also recognize this homolog protein.

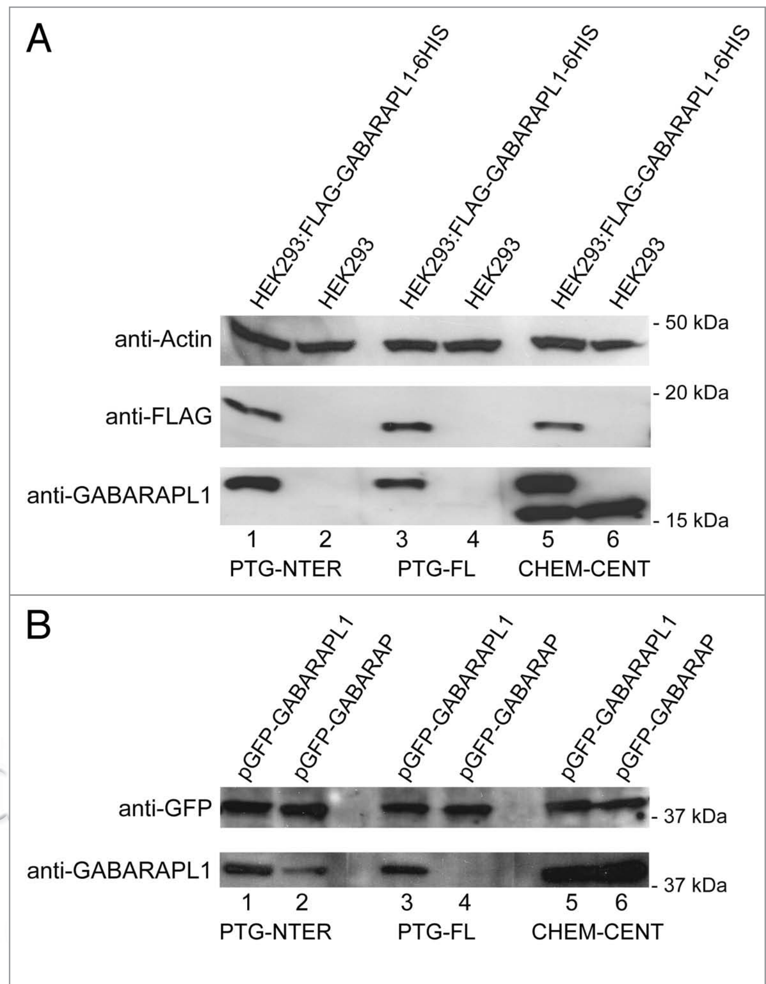

Figure 2. Western blot analysis of anti-GABARAPL1 antibodies specificity. Western blot analysis of HEK293 and HEK293:FLAG-GABARAPL1-6HIS cells (A) and HEK293 cells transiently transfected with a GFP-GABARAP or a GFP-GABARAPL1 vector (B). Whole cell lysates (20–40 μg) were loaded on a 12% or 15% SDS-PAGE and protein gel blotting experiments were performed using three different anti-GABARAPL1 antibodies directed against the N-terminus (PTG-NTER), the full-length protein (PTG-FL) or the center of the protein (CHEM-CENT) and control antibodies (anti-GFP, anti-FLAG M2 and anti-Actin). A representative experiment of 3 performed is shown.

Discussion

In this work, we studied the characteristics of three different anti-GABARAPL1 antibodies in protein gel blot and immunohistochemical analysis. With the exception of the anti-ATG8L antibody from Protein Tech Group (PTG-FL), these antibodies are described to be specific to GABARAPL1, implying that they do not recognize the closest homolog, GABARAP.

Using HEK293 cell lysates, we demonstrated that the antibodies tested displayed differing affinities for the denatured forms of GABARAPL1 and GABARAP. Of the antibodies that

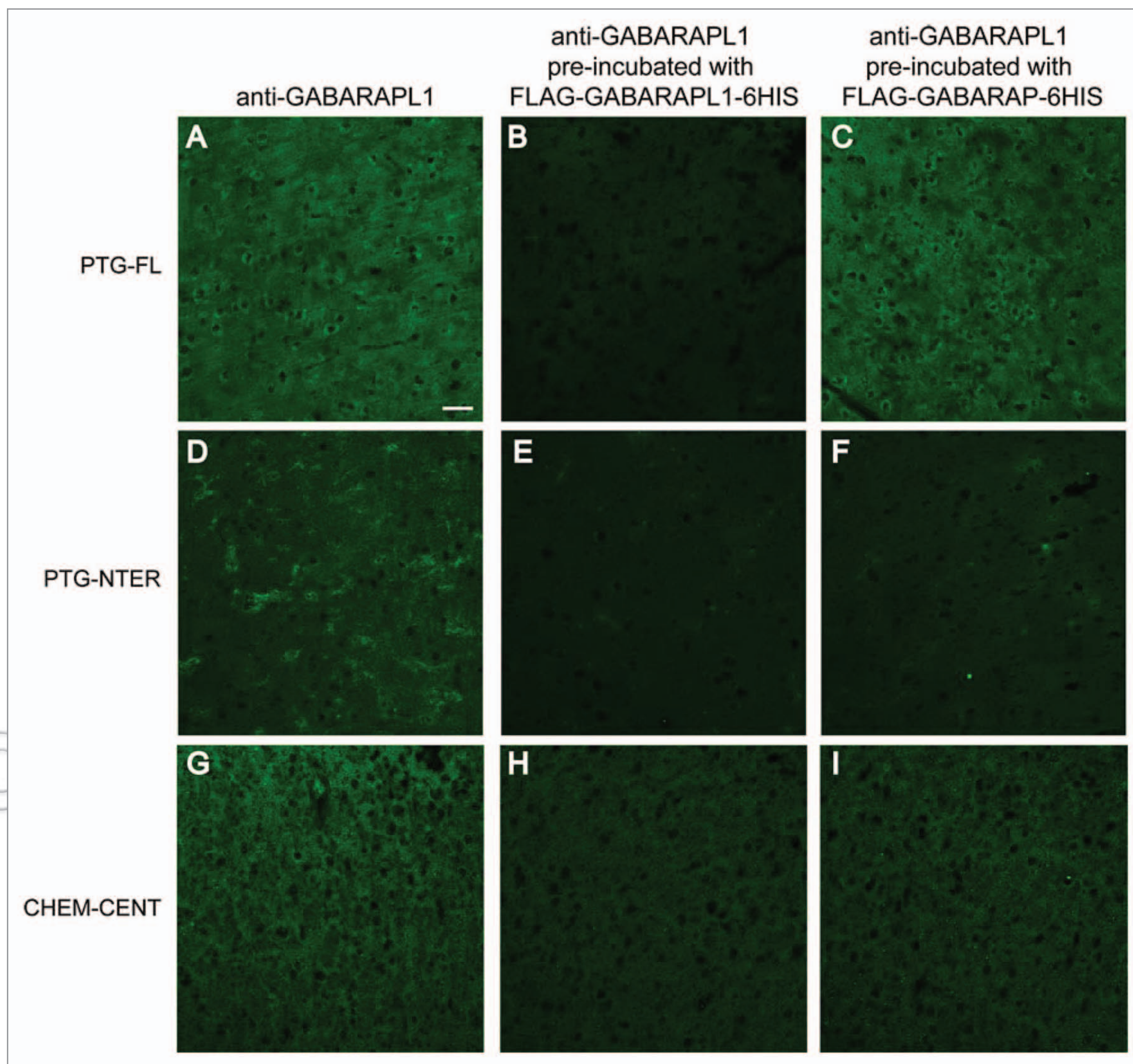

Figure 3. Immunohistochemical analysis of blocking protein experiments. Immunohistochemical analysis of rat brain tissue with anti-GABARAPL1 antibodies. One μl of pure anti-GABARAPL1 antibody was pre-incubated in either PBST alone, 0.5 nmol FLAG-GABARAP-6HIS in PBST or 0.5 nmol FLAG-GABARAPL1-6HIS in PBST for 2 h before incubation on rat brain sections overnight as described in the material and methods. Scale bar represents 20 μm.

we chose, the antibody raised against a peptide sequence within the central part of the GABARAPL1 protein was the least efficient at discerning between the two proteins but, surprisingly, this antibody displayed a higher affinity for these proteins. Finally, the antibodies, raised against the N-terminus and the full-length protein, appear to be able to distinguish between the two homolog proteins under the conditions used. It is worth noting, however, that even these antibodies have, under other conditions, recognized the recombinant GABARAP protein in protein gel blotting experiments[16] and one must therefore be vigilant of the protocol used when using these antibodies for GABARAPL1 detection. Our protein gel blot experiments indicated that the PTG antibodies seem to be specific of GABARAPL1 but when we analyzed their specificity in vivo, we observed some differences. Indeed, when we pre-incubated each antibody with either GABARAP or GABARAPL1 before performing an immunohistochemistry staining on mouse brain sections, we observed that only the PTG-FL antibody showed a specific staining, which was inhibited by a pre-incubation with the GABARAPL1 protein and not the GABARAP protein. All together, our results demonstrate that only the PTG-FL antibody seems to be specific of GABARAPL1 in vitro and in vivo.

In the past, our studies have utilized alternatives to endogenous protein detection such as mRNA expression or overexpression of a recombinant GABARAPL1, due to the lack of available antibodies. The identification of only one of the two homologs has been limited, making it difficult to associate the cellular roles being examined to one protein or the other. Although the conclusions obtained regarding the fundamental function of these two molecules in the cells remain valid, there is some doubt about the real implication of each of these proteins separately in the roles identified.

To conclude, experimenters should be very careful when working with anti-GABARAP and GABARAPL1 antibodies for

both in vitro and in vivo detection since they most likely cross-react and detect both proteins simultaneously. It would be judicious to either first demonstrate the degree of specificity of the antibody or intentionally target the two proteins together, as has previously been demonstrated for siRNA experiments.[17] In the future, the production of specific monoclonal antibodies would be more than useful to further characterize the function of each protein independently in vivo, as they have been described as playing an important role in various cellular and physiological processes such as apoptosis, autophagy, brain receptor transport or tumor progression.

Materials and Methods

Animals. All animal care and experimental protocols adhere to the institutional guidelines. Cerebral tissue from an adult male Sprague Dawley rat was used for competing protein experiments.

Vectors. To construct the psBET-FLAG-GABARAP-6HIS and psBET-FLAG-GABARAPL1-6HIS vectors, *gabarap* and *gabarapl1* cDNA were amplified by polymerase chain reaction (PCR) from the pcDNA3.1-*gabarap* and pcDNA3.1-*gabarapl1*,[18] plasmids using the following primers: 5'-AAA TCT AGA ATG AAG TTC GTG TAC AAA GA-3' and 5'-TTT GGA TCC CAG ACC GTA GAC ACT TTC GT-3' for *gabarap* and 5'-AAA TCT AGA ATG AAG TTC CAG TAC AAG GA-3' and 5'-TTT AGA TCT CTT CCC ATA GAC ACT CTC AT-3' for *gabarapl1*. The protocol used the following settings: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min and one final extension at 72°C for 10 min. The reactions were performed in a 25 µl volume containing 0.2 µM of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 15 ng of template DNA and 1 U of Go Taq DNA Polymerase (Promega, M3171). The PCR fragments were then purified using the NucleoSpin Extract II Kit (Macherey-Nagel, 740609.10) and ligated into the pGEM-T easy vector (Promega, A1360) using a T4 DNA ligase (Promega, M1801) according to the manufacturer's protocol. The *gabarap* and *gabarapl1* cDNA were then digested with *XbaI* (Fermentas, FD0684) and BamHI (Fermentas, FD0054) or BglII (Fermentas, FD0083), respectively and subcloned into the psBET-Flag/6His vector[19] to produce the psBET-FLAG-GABARAP-6HIS and psBET-FLAG-GABARAPL1-6HIS vectors, respectively. Plasmid sequences were confirmed by DNA sequencing using the previously described method in reference 20 (3130 Genetic Analyzer, Applied Biosystems).

Recombinant protein expression and purification. BL21-DE3 *E. coli* transformed with psBET-FLAG-GABARAP-6HIS or psBET-FLAG-GABARAPL1-6HIS were grown in 100 ml Luria-Bertani (LB) broth in the presence of 100 µg/ml kanamycine at 37°C. Protein expression was induced with 0.5 mM Isopropyl-β-D-Thiogalactopyranoside (IPTG) for 2 h. Bacteria were then pelleted at 5,000 g for 10 min at 4°C and resuspended in 5 ml of Sonication buffer (20 mM TRIS-HCl, pH 7.2–7.3, 20% Glycerol, 0.2 mM EDTA pH 8.0, 500 mM KCl, 10 mM Imidazole, 10 mM β-EtSH, 1 mg/ml lysozyme) per gram of bacteria. The bacterial solution was then incubated on ice for 30 min and sonicated three times for 15 sec (Sonics and Materials) followed by a second centrifugation at 20,000 g for 30 min at 4°C to clear the lysates. The FLAG-GABARAP-6HIS and FLAG-GABARAPL1-6HIS recombinant proteins were then purified using a Ni-NTA Agarose column Purification System (Qiagen 30210) according to the manufacturer's protocol. Briefly, protein lysates were incubated with 500 µl of packed Ni-NTA resin per 500 ml bacterial culture for 2 h at 4°C. The resin was then centrifuged at 5,000 g for 5 min at 4°C and washed three times with at least two volume equivalents of Wash buffer [20 mM TRIS-HCl, pH 7.2–7.3, 20% Glycerol, 0.2 mM EDTA pH 8.0, 100 mM KCl, 20 mM Imidazole, 10 mM β-EtSH, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] followed by a centrifugation at 10,000 g for 1 min at 4°C. The purified protein was eluted by incubation with two volume equivalents of Elution buffer (20 mM TRIS-HCl, pH 7.2–7.3, 20% Glycerol, 0.2 mM EDTA pH 8.0, 100 mM KCl, 10 mM β-EtSH, 0.5 mM PMSF) containing increasing concentrations of Imidazole (100–500 mM). Recombinant protein concentration was determined by comparison to a range of known BSA quantities on a 12.5% SDS-PAGE followed by Coomassie staining.

Cell culture. HEK293 (Human Embryonic Kidney) and HEK293:FLAG-GABARAPL1-6HIS cells were cultured in DMEM (Dulbecco's Minimum Essential Medium, PAA, E15-891) supplemented with 2 mM Stable L-Glutamine (PAA, M11-006), 100 µg/ml penicillin, 100 µg/ml streptomycin (Invitrogen, 15140) and 10% fetal bovine serum (FBS, PAA, A15-101) in a 5% CO_2 incubator at 37°C. Transient transfection of the HEK293 cells plated in a 6-well plate was accomplished with 500 ng of either pGFP-GABARAP or pGFP-GABARAPL1 and 1 µl of TransFast reagent (Promega, E2431) per well, following the manufacturer's instructions.

Tissue fixation and cryoprotection. Animals were anaesthetized by an intraperitoneal (i.p.) injection of 7% chloral hydrate (ROTH, K318.3) and perfused as previously described in reference 21, with 0.9% NaCl followed by ice-cold 1% paraformaldehyde (PFA, ROTH, 0335.4) fixative in PBS (0.137 M NaCl, 3.3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Extracted brains were subsequently post-fixed in 1% PFA for several hours at 4°C and cryoprotected by saturation in a 15% sucrose solution (Sigma, 17 994-9) at 4°C overnight. Tissues were frozen on dry ice in OCT (Tissue Tek 4583), sectioned into 10 µm sections at -20°C using a cryostat-microtome (Microm, HM560) and placed on gelatin-coated slides (ROTH, 4275.1) and stored at -40°C.

Immunohistochemistry. For immunohistochemical staining, slides were prepared by rehydrating in PBS-T (PBS with 0.3% Triton X100) for three 5-min washes at room temperature. Tissue was subsequently incubated with the appropriate primary antibody dissolved in milk diluant (PBS-T containing 1% bovine serum albumine, 10% lactoproteins and 0.01% sodium azide) overnight at room temperature. Primary antibodies used include: anti-ATG8L (PTG-FL, rabbit polyclonal, Proteintech, 11010-1-AP, 1:200), anti-GABARAPL1 (PTG-NTER, rabbit polyclonal, Proteintech, 18721-1-AP, 1:200), anti-GABARAPL1 (CHEMCENT, rabbit polyclonal, Chemicon Millipore, AB15278, 1:200). Slides were then washed three times with PBS-T for 5 min each at room temperature before being incubated with the

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appropriate secondary antibody diluted in milk solution for 1 h at room temperature. The secondary antibody used was the Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A11034, 1:800). Finally, the slides were washed with PBS-T three times for 5 min and coverslips were mounted with 70:30 glycerol:PBS-T.

Competing protein experiments. For immunohistochemical protein competing experiments, antibodies were incubated with either 0.05 or 0.5 nmol of purified recombinant protein (FLAG-GABARAP-6HIS or FLAG-GABARAPL1-6HIS) per 1 μl of pure antibody (either anti-ATG8L from Proteintech at 0.247 μg/μl, anti-GABARAPL1 from Proteintech at 0.773 μg/μl, or anti-GABARAPL1 from Chemicon at 0.62 μg/μl) diluted in PBS-T experiments for 2 h prior to detection experiments as described above.

Confocal microscopy. Tissue sections were examined with a fluorescence laser scanning confocal microscope Fluoview FV1000 BX (Olympus, France) and images were captured with a 40x/0.75 NA objective and a DP 75 numeric camera using the Fluoview FV1000 software (Olympus, France). All figures were assembled using Adobe Illustrator CS software.

Western blot analysis. Cells were lysed using the following lysis buffer: [50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, protease inhibitor cocktail (Sigma, P8340)] then 20–40 μg of cell lysates were resolved on a 12% or 15% polyacrylamide gel in running buffer (25 mM Tris base, 200 mM glycine and 0.1% SDS) at 120 V, using a Biorad Power Pack 1000. Proteins were transferred onto Immun-Blot PVDF 0.2 μm membranes (Biorad, 1620177) for 1 h at 4°C at 100 V in protein gel blot transfer buffer (25 mM Tris base, 200 mM glycine and 10% methanol). Membranes were subsequently blocked in TBS-T (199 mM TRIS-HCl, pH 7.4, 1.36 mM NaCl, 0.1% Tween 20) with 5% skim milk powder. Membranes were blotted with antibodies diluted in TBS-T supplemented with 0.5% skim milk against ATG8L (PTG-FL, rabbit polyclonal, Proteintech, 11010-1-AP, 1:2,000), GABARAPL1 (PTG-NTER, rabbit polyclonal, Proteintech, 18721-1-AP, 1:2,000), GABARAPL1 (CHEM-CENT, rabbit polyclonal, Chemicon Millipore, AB15278, 1:2,000), GFP (rabbit polyclonal, Millipore, AB3080, 1:5,000), FLAG M2 (mouse monoclonal, Sigma, F1804, 1:1,000) and Actin (rabbit polyclonal, Sigma, A5060, 1:3,000). The secondary antibody horseradish peroxydase-coupled anti-rabbit IgG was prepared in TBS-T containing 0.5% skim milk powder. Binding of antibodies to membranes was detected by Enhanced Chemiluminescence Plus Reagent (ECL Plus, GE Healthcare Life Sciences, RPN2132), according to the manufacturer's protocol.

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