SPATIO-TEMPORAL EXPRESSION ANALYSIS OF THE CALCIUM-BINDING PROTEIN CALUMENIN IN THE RODENT BRAIN

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Abstract—Calumenin is a Ca2+-binding protein that belongs to the CREC superfamily. It contains six EF-hand domains that exhibit a low affinity for Ca2+ as well as an endoplasmic reticulum retention signal. Calumenin exhibits a broad and relatively high expression in various brain regions during development as demonstrated by in situ hybridization. Signal intensity of calumenin is highest during the early development and then declines over time to reach a relatively low expression in adult animals. Immunohistochemistry indicates that at the P0 stage, calumenin expression is most abundant in migrating neurons in the zones around the lateral ventricle. In the brain of adult animals, it is expressed in various glial and neuronal cell types, including immature neurons in subgranular zone of hippocampal dentate gyrus. At the subcellular level, calumenin is identified in punctuate and diffuse distribution mostly in somatic regions where it co-localizes with endoplasmic reticulum (ER) and partially Golgi apparatus. Upon subcellular fractionation, calumenin is enriched in fractions containing membranes and is only weakly present in soluble fractions. This study points to a possible important role of calumenin in migration and differentiation of neurons, and or in Ca2+ signaling between glial cells and neurons.

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Key words: calcium-binding protein, cellular and subcellular distribution, calcium signaling, brain development, progenitor cells, glial cells.

Calumenin belongs to the CREC family of Ca2+-binding proteins, together with reticulocalbin, reticulocalbin-3, ERC-55, crocalbin, Cab45, and its splice variant Cab45b (Honore and Vorum, 2000; Honore, 2009). Calumenin contains six EF-hand helix-loop-helix motifs with low affinity for Ca2+ and harbors an endo(sarco)plasmic reticulum (ER) HDEF retention signal located at its C-terminus (Yabe et al., 1997; Vorum et al., 1998; Honore and Vorum, 2000; Honore, 2009). This signal has a lower efficiency for ER retention as compared with the well studied KDEL sequence, explaining why calumenin was not only described to participate in the secretory pathway as other CREC family members, but is also found extracellularly in cultured human fibroblasts (Vorum et al., 1999; Honore, 2009). Previous studies have demonstrated expression and distribution of calumenin in non-neuronal tissues; however, there are no reports describing the possible expression of calumenin in the rodent CNS so far.

Neuronal Ca2+-binding proteins exert effects on a variety of important physiological functions, such as activation of transcription factors, neuronal growth and survival, neurotransmitter release, channel and receptor regulation, and synaptic plasticity (Burgoyne, 2007; Chaumont et al., 2008; Amici et al., 2009). The importance of Ca2+-signaling in glia has also been well documented. It is believed to be a form of glial excitability, enabling glial cells to integrate extracellular signals, communicate with each other, and exchange information with neurons (Verkhratsky, 1997; Metea and Newman, 2006). Like in other cells, ER Ca2+-storage and -release in brain cells contribute crucially to Ca2+ signals, while ER Ca2+-binding proteins provide a high capacity buffering mechanism, which results in the lowering of the concentration of free Ca2+ in the ER (Koch, 1990; Verkhratsky, 2004, 2005). It has been previously described that calumenin is abundant in ER of diverse non-neuronal cell types (Yabe et al., 1997; Tyedmers et al., 2005; Sahoo and Kim, 2008, 2010; Sahoo et al., 2009). However, a possible localization of calumenin in the neuronal or glial ER has not been confirmed so far.

Calumenin was also described in functional association with the cytoskeleton where it is translocated from the organelle-containing membrane fraction to the cytosol upon mitotic arrest and during late apoptosis in HeLa cells, treated with the tubulin-depolymerizing drug taxol (Bull et al., 2010). Additionally, extracellular calumenin appears to have an autocrine and/or paracrine modulating effect on organization of the actin cytoskeleton (Ostergaard et al., 2006). Whether calumenin shows cytoplasmic and/or extracellular localization in the CNS and whether it plays a role in any of the essential cytoskeletal functions in neurons (Schaar and McConnell, 2005; Hotulainen and Hoogenraad, 2010; Svitkina et al., 2010), including neuronal growth, migration, synaptogenesis, and synaptic plasticity, remains to be elucidated.

As a basis for further investigations on putative functions of calumenin in the brain, this study provides the first...
comprehensive spatio-temporal characterization of calumenin expression in the CNS.

EXPERIMENTAL PROCEDURES

All the chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA, or from Invitrogen, San Diego, CA, USA, unless otherwise stated.

In situ hybridization

For in situ hybridization, an 827 bp long fragment of the calumenin sequence (nr 1529–2355) was amplified from the whole mouse brain cDNA library (a gift from Han Kyu Lee, ZMNH), and the resulting PCR product was cloned into pBluescript KS (-) vector (Stratagene, Santa Clara, CA, USA) using EcoRI and XhoI restriction sites. The correct sequence of the insert was confirmed by sequencing. Radioactively labeled RNA probes were prepared using the MAXIscript in vitro transcription kit (Ambion, Austin, TX, USA) in the presence of α-35S-UTP. Probes were purified with ProbeQuant sephadex G-50 microcolumns (Amersham, Buckinghamshire, UK). In situ hybridization was performed as previously described (Fehr et al., 1987). Three independent experiments with three sets of animals for each time point were used. The sense transcription product served as a negative control.

Extraction of proteins

All the animals were provided by the Animal Facility of University Clinics “Eppendorf” in Hamburg, or by the Department of Animal Care and Genetics—Himberg, Medical University of Vienna. Buffers were supplemented with Complete Mini Protease inhibitor cocktail (Roche, Mannheim, Germany). Preparation of membranes from different organs was performed using IM-Ac buffer (20 mM HEPES, 100 mM K+ -acetate, 40 mM KCl, 5 mM EGTA, 5 mM MgCl2), as previously described (Maas et al., 2006). Mice were sacrificed either at the day of birth (P0) or at adult age (12–16 weeks old), and their organs were dissected and placed in an ice-cold IM-AC buffer. Homogenization was performed using a Dounce-homogenizer with a teflon pestle for 10 up-and-down strokes at 1,000 rpm. The homogenates were ultracentrifuged at 150,000 ×g, and the supernatants were discarded. The pellets were then once more washed in IM-Ac buffer and centrifuged as described before. The resulting pellets were finally suspended in appropriate volumes of IM-Ac buffer, and the protein concentrations of the samples were determined using a BCA kit (Pierce, Bonn, Germany). Samples for SDS-electrophoresis were prepared using Nu-PAGE sample buffer (Invitrogen, San Diego, CA, USA).

For preparation of membranes of different brain regions, P0, P7, P14, or P21 mice were decapitated, and their cortex, hippocampus, and cerebellum were dissected in ice-cold phosphate buffered saline (PBS; 136.9 mM NaCl, 2.68 mM KCl, 1.8 mM KH2PO4, 10 mM Na2HPO4, pH 7.4). The samples for SDS-PAGE were then prepared in the same way as described for other tissues, using IM-Ac buffer.

For neuronal extracts, cultured neurons were prepared as previously described (Maas et al., 2006). Cells were harvested in lysis buffer (PBS, 1% Triton X-100) supplemented with Complete Mini Protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min on ice. Lysates were centrifuged at 1,000 ×g for 5 min at 4 °C, and the supernatant was recovered. Protein concentration was determined using a BCA assay kit (Pierce, Bonn, Germany). Subsequently, protein samples were subjected to SDS-PAGE and Western blotting.

Differential centrifugation

Differential centrifugation was performed as previously described (Maas et al., 2006). In brief, P10 rats were decapitated, and the brains were put into a homogenization buffer (320 mM sucrose, 10 mM HEPES/KOH, pH 7.9, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA) and pottered using a glass-telfon-doncuer. Extracts were centrifuged at 1,000 ×g for 15 min. The resulting pellet was designated as P1. The supernatant, S1, was further centrifuged at 10,000 ×g for 30 min, and the resulting pellet was named P2. The remaining supernatant S2 was centrifuged at 100,000 ×g for 1 h, to obtain pellet P3, with the supernatant referred to as S3. Protein contents were determined using a BCA protein assay kit (Pierce, Bonn, Germany).

Specificity of the anti-calumenin antibody

To test whether rabbit anti-calumenin antibody (Cat. Nr HPA006018, Atlas Antibodies, Stockholm, Sweden) specifically recognizes calumenin, an antibody-competition experiment was performed. For that, the coding sequence of calumenin was amplified from the whole mouse brain cDNA library (a gift from Han Kyu Lee, ZMNH), and the resulting PCR product was cloned into pCMV6-AC vector (Origene, Rockville, MD, USA) using EcoRI and XhoI restriction sites. The correct sequence of the insert was confirmed by sequencing at VBC-Biotech Service GmbH, Vienna, Austria. The pCMV6-AC plasmid DNA encoding for calumenin, or an empty vector (for mock transfections), were used for transient transfection of HEK 293 cells, as previously described (Shrivastava et al., 2011). Briefly, HEK 293 cells (CRL 1573, American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco’s Modified Eagle’s Medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (BioWhittaker, Lonza, Basel, Switzerland), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin G, and 100 μg/ml streptomycin in 75 cm2 culture dishes using standard cell culture techniques. HEK 293 cells (3 × 105) were transfected with a total amount of 15 μg of cDNAs using the calcium phosphate precipitation method (Chen and Okayama, 1988). The cells were harvested 48 h after transfection. Subsequently, the proteins were extracted from the harvested cells by using 1% Triton X-100 containing PBS buffer, supplemented with “Complete” protease inhibitors (Roche, Mannheim, Germany). The anti-calumenin antibodies were incubated for 4 h at 4 °C either with HEK 293 cell extract expressing calumenin, or with extract of mock-transfected HEK 293 cells. The mixture of antibodies and the HEK 293 cell extract was then added to the blocking buffer (composition described in sections “Western blotting,” “Immunohistochemistry,” and “Immunocytochemistry” under Experimental procedures, depending on the application) and was used for downstream application—either Western blotting, immunohistochemistry, or immunocytochemistry. Although there is presumably endogenous expression of calumenin in human embryonic kidney cells (as also shown in our experiments for embryonic mouse kidney), it could be expected that specific anti-calumenin antibodies would be blocked to a significantly higher extent by overexpressed than by endogenous calumenin, resulting in a significant reduction of signal on using these antibodies. Since that was the case, calumenin was obviously recognized by this antibody. Although a possible cross-reactivity of the antibody with an additional protein cannot be excluded, it seems to be very unlikely (see “Immunohistochemical distribution of calumenin in P0 and adult mouse brain,” under Results section).

Western blotting

SDS-PAGE was performed as previously described (Sarto-Jackson et al., 2007) using gels containing 10% polyacrylamide in a discontinuous system. The same amount of protein was applied to each lane. Proteins were separated on the gels and were then tank-blotted onto prewetted polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). After blocking with 1.5% nonfat dry milk powder in PBS and 0.1% Tween 20 for 1 h at room temperature, the membranes were incubated overnight with primary
antibodies diluted in blocking buffer at 4 °C. The membranes were extensively washed and incubated with secondary antibodies (anti-rabbit, or anti-mouse-alkaline phosphatase, F(ab')2 fragments, Jackson ImmunoResearch, Suffolk, UK) for 1 h at room temperature. PVDF membranes were again washed extensively as described previously and then equilibrated in assay buffer (25 mM diethanolamine and 1 mM MgCl₂, pH 10.0) for 15 min, and secondary antibodies were visualized by the reaction of alkaline phosphatase with CDP Star (Applied Biosystems, Bedford, MA, USA). The chemiluminescent signal was quantified by densitometry after exposing the immunoblots to the Fluor-S Multimager (Bio-Rad) and evaluated using the Quantity One software. The sum of intensities of all specific bands on a blot was assigned value 100, and individual band intensities were transformed into relative numbers accordingly. In agreement with previous reports (Ferguson et al., 2005) it was noticed that the amount of "housekeeping" proteins such as actin or pan-cadherin changed at different time points and in different organs in spite of applying the same amount of protein to each lane of the gel. Therefore signals for actin were only used for normalizing data from the same tissue and time point.

Polycional rabbit anti-calumenin antibodies were purchased from Atlas Antibodies (Cat. Nr HPA006018), Stockholm, Sweden, and were used at a dilution of 1:400. Anti-actin antibodies (Sigma, St. Louis, MO, USA) were used at a dilution of 1:2000. Anti-PSD-95 antibodies (BD Biosciences, Bedford, MA, USA) were used at a dilution of 1:250.

Immunohistochemistry

Experiments were performed on three adult C57BL/6 male mice. The mice were deeply anesthetized and perfused through the ascending aorta with 0.9% NaCl, followed by a mixture of 4% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer (PB; pH 7.2–7.4). Brains were removed, extensively rinsed in PB, and serially sectioned in the coronal or sagittal plane on a vibratome at 50 μm thickness. Sections were kept in PB containing 0.05% sodium azide.

Immunofluorescence experiments were carried out according to previously published procedures (Klausberger et al., 2003). Briefly, free-floating sections were rinsed in 0.1 M PB and incubated in 0.1% Triton X-100 in PB. Subsequently, the sections were blocked by incubation in 20% normal horse serum diluted in Tris-buffered saline (TBS; 50 mM Tris, pH 7.2, 0.85% NaCl) for 2 h and transferred into solutions containing mixtures of primary antibodies (rabbit anti-calumenin antibody, Atlas Antibodies, Stockholm Sweden, dilution 1:100; mouse monoclonal anti-NeuN antibody, Millipore, MA, USA, dilution 1:1000; mouse monoclonal anti-Calbindin D28k antibody, Marly, Switzerland, dilution 1:10; chicken polyclonal anti-GFAP antibody, Abcam, Cambridge, UK, dilution 1:3000; mouse monoclonal PSA-NCAM antibody, Chemicon, Millipore, MA, USA, dilution 1:400) for 18–24 h at room temperature. The next day, sections were washed and incubated in appropriate secondary antibodies conjugated either with Alexa Fluor 488 (anti-rabbit; Invitrogen Molecular Probes, dilution 1:3000) or indocarbocyanine (anti-mouse Cy3; Jackson Immunoresearch Laboratories Inc., diluted 1:400) for 4 h. All antibodies were diluted in TBS containing 0.1% Triton X-100 and 1% normal horse serum. After washing in 50 mM Tris buffer, pH 7.4, sections were mounted in Aqua PolyMount (Polysciences Europe, Eppelheim, Germany) and imaged with a Leica TCS SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). All antibodies were tested for optimal dilution, and secondary antibodies were tested for cross-reactivity and non-specific staining. DAPI nuclear marker was purchased from Invitrogen, San Diego, CA, USA.

Immunocytochemistry

Cultured hippocampal neurons were prepared as previously described (Maas et al., 2006). In brief, 12 mm sterile glass coverslips were coated with poly-L-lysine (50 μg/ml) and laminin (20 μg/ml). Hippocampi of eight newborn rats (P0) were dissected in PBS/10 mM glucose and subsequently trypsinized for 25 min at 37 °C. The neurons were then triturated through fire polished Pasteur pipettes of two different diameters and plated on previously coated and washed coverslips at a density of about 30,000 cells/cm² in plating medium (DMEM/F12, 10% fetal calf serum (v/v), 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin). The cells were grown for 3–5 h at 37 °C and 5% CO₂. At this time point, the plating medium was replaced with 1 ml neuronal culture medium (Neurobasal medium, 25 μg/ml pyruvate, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 2% B27 supplement (v/v)). At days in vitro (DIV) 12–14, the neurons were fixed in 4% paraformaldehyde/4% sucrose for 12 min. Subsequently, the cells were washed with PBS and permeabilized with 0.25% Triton X-100 containing PBS buffer for 4 min. Unspecific binding sites were blocked with 1% (w/v) bovine serum albumin (AppliChem, Darmstadt, Germany) in PBS (blocking buffer). Primary antibodies were applied in blocking buffer overnight at 4 °C, as previously described (Tagnou et al., 2007). The following primary antibodies were used: anti-KDEL (Stressgen, Ann Arbor, MI, USA) dilution 1:100, anti-GM 130 (BD Biosciences, Franklin Lakes, NJ, USA) dilution 1:100, anti-synaptophysin (Synaptic Systems, Göttingen, Germany) dilution 1:1000. Application of secondary antibodies was carried out in the blocking buffer, after washing of coverslips with PBS. Anti-rabbit-Alexa488 antibody (Invitrogen, San Diego, CA, USA) was applied at dilution 1:200, anti-mouse pig-Cy3 (Immunoresearch Laboratories, Inc, West Grove, PA, USA) at dilution 1:500, and anti-mouse-Cy3 secondary antibodies (Jackson Immunoresearch Laboratories, Inc, West Grove, PA, USA) at dilution 1:600. Fluorescence imaging was carried out with an inverted Leica TCS-SP5 II laser scanning confocal microscope. For simultaneous multichannel fluorescence, images were taken in a sequential channel recording mode.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The normality of the distribution of values within each group was tested by the Kolmogorov–Smirnov normality test. As the normal distribution of values was confirmed, the differences in signal intensities in Western blots were analyzed using parametric Student’s unpaired t-test. For P<0.05 (*) difference was designated as statistically significant, whereas P<0.01 represented highly significant difference (**).

RESULTS

Distribution of calumenin transcripts in mouse brain during development

To analyze calumenin mRNA distribution in the developing mouse CNS, a radioactive probe corresponding to calumenin nucleotides 1523–2355 was used (Fig. 1A). At embryonic day 12 (E12), a moderate hybridization signal in the developing CNS (Fig. 1B) as well as in other tissues: bones, skin, heart, lung, and muscles was observed (data not shown). At E12–E20 considerable levels of calumenin transcripts were observed in the neuroepithelium of the cortex, in cerebellum, as well as the brain stem (medulla oblongata, pons, and midbrain). Higher than average signal intensity was observed in the regions around aqueduct, fourth ventricle, and lateral ventricle (arrows), the regions representing germinal zones containing migrating neurons.
and glia cells (Hatten, 1999). Specificity of in situ hybridization signals was controlled by using a sense transcript of the same cDNA probe used for hybridization.

Subsequently, in situ hybridization of mouse brain tissue derived from different postnatal stages (P7, P14, and adult) was performed. A basal expression of calumenin mRNA was visible throughout the brain (Fig. 1C). Higher signal intensities were observed in the cerebellum, the hippocampal formation, the thalamus, the olfactory bulb, and the cortex of both P7 and P14 mice (Fig. 1C, arrows). However, the overall specific signal intensity decreased with age (Fig. 1C, adult), as compared with signals of the sense control. These data indicate that calumenin transcripts are well expressed in the second half of mouse embryonic development, suggesting that calumenin plays a role in early developmental stages.

Distribution of the calumenin protein in different mouse tissues during development

Calumenin was demonstrated to have important roles in different tissues, both under physiological and pathological
conditions (Tyedmers et al., 2005; Jung et al., 2006; Cho et al., 2009; Honoré, 2009; Westergaard Hansen et al., 2009). To investigate its distribution during development, the specificity of the anti-calumenin antibody used in this study was tested as a first step. The anti-calumenin antibody alone (data not shown), or pre-incubated with extracts of mock-transfected HEK 293 cells (Fig. 2A) labeled a single protein band with apparent molecular mass of 45 kDa (Sahoo and Kim, 2008; Sahoo et al., 2009) in HEK 293 cells transfected with recombinant calumenin or in P0 brain membranes. In untransfected (data not shown) or mock-transfected HEK 293 cells, a protein band with the same molecular mass was only weakly labeled, probably representing endogenous calumenin. This small amount of endogenous calumenin was obviously not sufficient to significantly reduce the labeling of these proteins by the anti-calumenin antibody. In contrast, staining of this protein band was no longer observed either in mock-transfected or calumenin-transfected HEK 293 cells, or in P0 brain membranes (Fig. 2A), when the antibody was pre-adsorbed by extracts of calumenin-transfected HEK 293 cells. These data indicate that the observed 45 kDa protein represented calumenin.

To assess the amount of calumenin protein in various tissues compared with its relative expression in brain, the same amount of membrane protein from mouse brain, heart, kidney, lung, and stomach of P0 and adult animals was subjected to SDS-PAGE and immunoblotting. As shown in Fig. 2B, the calumenin levels were significantly higher in P0 mice than in adult mice in all tissues investigated.

As a next step, the levels of calumenin in three different brain regions (cerebellum (Cb), cortex (Cx), and hippocampus (Hip)) were assessed during early postnatal development (P0, P7, P14, and P21, Fig. 3). Data obtained indicated a decreased expression of calumenin at the end of the third postnatal week in all tissues investigated. Overall, these data are consistent with the in situ hybridization data (Fig. 1) and suggest that calumenin protein levels gradually decrease from early postnatal development to adulthood.

Immunohistochemical distribution of calumenin in P0 and adult mouse brain

To investigate the distribution of calumenin in the mouse brain, we performed immunostaining of fixed sagittal and coronal sections of either P0 or adult mouse brain tissue. Although the signal for calumenin in adult hippocampus was lost upon pre-adsorption of the antibody with overexpressed calumenin from the HEK 293 cell extracts (Fig. 4A), the same antibody could still recognize its target upon incubation with the extract of mock-transfected HEK 293 cells (Fig. 4B, arrows). In addition, the distribution of the calumenin protein in P0 and adult brain, for instance in cerebellum and around the lateral ventricle, was similar to that observed for calumenin mRNA (Fig. 1). Finally, the restricted distribution of calumenin in adult brain tissue (Fig. 4H–Q) indicates that the antibody used specifically binds to its target. Although a crossreactivity of the anti-body with another protein cannot be completely excluded, all our data suggest that such a crossreactivity is highly unlikely.

As depicted in Fig. 4C, the signal intensity for calumenin in cerebral cortex, cerebellar cortex, mitral cell layer of olfactory bulb, and migrating cells in the germinal zones around the lateral ventricle of P0 mice was higher than in
Bergman glia cells, specialized cerebellar astrocytes, probably represent glia. Morphologically, they resemble Purkinje neurons—marker (Fig. 4J, red), these cells most were negative for NeuN and also for calbindin D28k, a large cell bodies of Purkinje neurons. Purkinje neurons, were situated in the PCL (arrows in Fig. 4G–J), between express post-mitotic neuronal marker NeuN (Fig. 4H, I, molecular layer (asterisk in Fig. 4H). These cells did not in the granule cell layer (crossed arrow in Fig. 4H) and 4F, arrow). By using confocal microscopy on sagittal (Fig. 4E, F, arrowhead), and deep nuclei of cerebellum (Fig. 4L), calumenin levels in cerebellum, cortex, and hippocampus, tend to decrease in the third postnatal week (Cb: P0, 11.8±2.9; P7, 12.0±2.4; P14, 11.1±0.9; P21, 7.9±1.3; Cx: P0, 8.7±0.6; P7, 10.5±1.6; P14, 8.5±0.6; P21, 5.7±0.5; Hip: P0, 7.9±1.7; P7, 9.9±2.2; P14, 5.9±0.6; P21, 2.9±1.1). The experiment was independently performed three times. For each experiment different animals were used, one for each time point. Three Western blots were done per each experiment. Asterisk represents statistically significant differences (*P<0.05). Key: Cb, cerebellum; Cx, cortex; Hip, hippocampus; n.s., non-significant.

other brain regions. As seen in higher magnification (Fig. 4D), calumenin immunoreactivity could be observed in cell bodies of immature migrating cells around the lateral ventricle in P0 mice (arrows in Fig. 4D).

After immunohistochemical staining of sagittal sections of adult mouse brains with anti-calumenin antibody, a moderate signal was detected in all brain regions (Fig. 4E). However, a significant immunoreactivity was observed in olfactory tract, thalamic nuclei and Purkinje cell layer (PCL, Fig. 4E, F, arrow), and deep nuclei of cerebellum (Fig. 4F, arrow). By using confocal microscopy on sagittal (Fig. 4G) and coronal (Fig. 4H–J) cerebellar sections, individual calumenin-positive cells (stained in green) were detected in the granule cell layer (crossed arrow in Fig. 4H) and molecular layer (asterisk in Fig. 4H). These cells did not express post-mitotic neuronal marker NeuN (Fig. 4H, I, red). The majority of calumenin-positive cells in cerebellum were situated in the PCL (arrows in Fig. 4G–J), between large cell bodies of Purkinje neurons. Purkinje neurons, however, did not show calumenin staining (arrowheads in 4H, I). Given that calumenin-positive cells in PCL (green) were negative for NeuN and also for calbindin D28k, a Purkinje neurons-marker (Fig. 4J, red), these cells most probably represent glia. Morphologically, they resemble Bergman glia cells, specialized cerebellar astrocytes hav-
Fig. 4. Tissue distribution of calumenin protein in brain sections of newborn (P0) and adult mice. (A) Coronal section of the hippocampal dentate gyrus stained with anti-calumenin antibody pre-adsorbed by extracts of calumenin-transfected HEK 293 cells, showing no signal. (B) Coronal section of the hippocampal region stained with anti-calumenin antibody pre-adsorbed by extracts of mock-transfected HEK 293 cells, showing specific signal in individual cells of subgranular zone and granule cells layer of the dentate gyrus (arrows). (C) Whole brain sagittal section of a P0 mouse brain stained with anti-calumenin antibody. Calumenin displays widespread moderate protein expression with higher signal intensity in cerebellum (Cb), cortex (Cx), olfactory bulb (BO), and migrating cells (MC) around lateral ventricle. (D) Confocal image of the region around the lateral ventricle of P0 mouse brain. High immunoreactivity of calumenin is detected in migrating cells and radial-like glia around the lateral ventricle (arrows). (E) Whole brain sagittal section of an adult mouse brain stained with anti-calumenin antibody. Calumenin protein is moderately expressed throughout the brain. The strongest signal intensity is detected in cerebellum (Cb), olfactory tract (TBO), and thalamic nuclei (Th). (F) Stereomicroscopic image of calumenin immunoreactivity in adult mouse cerebellum. The highest expression of calumenin is visible in Purkinje cell layer (arrowhead) and deep nuclei of cerebellum (arrow). (G) Confocal image of a sagittal adult mouse cerebellar section co-stained for calumenin (green) and a nuclear marker DAPI (blue). The immunoreactivity of calumenin is strongest in PCL (arrow). Some of calumenin-positive cells of PCL send their extensions throughout the molecular cell layer, which is a morphological characteristic of Bergman glia cells (red arrowhead). (H) Confocal image of coronal cerebellar cortex section stained for calumenin (green) and NeuN (red), a postmitotic neuronal marker. The cells in PCL having the strongest signal intensity for calumenin are NeuN-negative (arrow). There are also individual NeuN-negative cells in ML (asterisk) and GCL (crossed arrow) that are moderately positive for calumenin. The arrowhead indicates a large body of a Purkinje cell. (I) Enlarged image of the boxed region from (H)—a group of cells strongly expressing calumenin (arrow) next to the large calumenin-negative cell body of Purkinje cell (arrowhead). (J) Confocal image of cerebellar cortex; calumenin (green), calbindin D28k (red). Calumenin is expressed by cells in PCL (arrow) that send their extension through ML (red arrowhead), which is typical for Bergmann glia. Purkinje cells express calbindin D28k, but no calumenin. (K) Olfactory bulb. A sagittal section was stained for calumenin (green) and nuclear marker DAPI (blue). Individual cells in external plexiform layer are moderately positive for calumenin (arrowhead). (L) Coronal section of pons. Calumenin (green) and NeuN (red) colocalize in individual large neuronal cell bodies (arrow), but there are also NeuN-negative small cells expressing calumenin (arrowhead). (M) Coronal section of the dentate gyrus co-stained for calumenin (green) and NeuN (red). In the basal part of granule cell layer (DGL) there are both NeuN-positive and negative cells of diverse morphology strongly stained for calumenin (arrow). In molecular (DML) and polymorphic layer (PoDG) however, there are only small, individual NeuN-negative cells expressing calumenin (arrowheads). (N) Co-staining of dentate gyrus for calumenin (green) and glial fibrillary acidic protein (GFAP), a marker of astrocytes and neuronal progenitor cells (red). Arrows point to calumenin-positive cells in subgranular zone that co-express GFAP, whereas arrowheads point to astrocytes in DGL and DML, also expressing calumenin. (O) Co-staining of dentate gyrus for calumenin (green) and PSA-NCAM, early neuronal marker (red). Some of calumenin-positive cells in subgranular zone also express PSA-NCAM (arrows). (P) Image of a dentate gyrus section stained for calumenin (green) and NeuN, a postmitotic neuronal marker (red). Arrow points at a large cell in subgranular layer expressing both calumenin and NeuN (weak expression). One cell expressing both proteins is depicted in boxed region at the border between DGL and DML. (Q) Enlarged image of the cell in the boxed region from (P). Arrow points to a weak NeuN signal in the nucleus, whereas arrowhead represents the somatic calumenin staining. Key: BO, olfactory bulb; Cb, cerebellum; Cx, cortex; DG, dentate gyrus; DML, molecular layer of dentate gyrus; GR, granule cell layer of olfactory bulb; EP, external plexiform layer; GCL, granule cell layer of cerebellum; HC, hippocampus; LV, lateral ventricle; MB, mitral cell body layer; MC, migratory cells; ML, molecular layer of cerebellum; P5, pons; PCL, Purkinje cell layer; TBO, olfactory tract; Th, thalamus; PoDG, polymorphic layer of dentate gyrus. Scale bars: (A, B) 50 μm, (C) 1 mm, (D) 0.5 mm, (E, F) 1 mm, and (G–P) 50 μm.
et al., 2009). To investigate whether calumenin co-localizes with membrane structures of neuronal tissue, a differential centrifugation of adult rodent whole brain lysates was performed. Immunoblotting of individual subcellular fractions revealed strong signals representing calumenin in lanes containing the P1 pellet, which consists of nuclei and remaining intact cells (data not shown). Furthermore, the P2 pellet, representing the plasma membrane fraction (Lee, 2001), and the P3 pellets, consisting of small membranes and large vesicular structures (Kennedy, 1997; Saito et al., 1997), contained significant amounts of calumenin (Fig. 5). In contrast, smaller amounts of calumenin were detected in the supernatants S2 and S3 representing the cytoplasmic fractions typically enriched in soluble proteins. Detection for PSD-95 was used as a positive control, because this protein has been analyzed previously upon
specificity of the calumenin signal obtained. We detect its target anymore. This again demonstrated the over-expressed calumenin (Fig. 6B, left), in contrast to and different subcellular markers. Control experiments in-12–14 were co-stained with antibodies against calumenin and various subcellular fractionation (Lee, 2001) and is known to be enriched in P2 fractions (Kennedy, 1997) and intracellular cargo vesicles (P3) (Mok et al., 2002).

Subcellular localization of calumenin in hippocampal cell culture

It has been reported previously that calumenin is present throughout the secretory pathway of some mammalian cells (Vorum et al., 1999). To study the subcellular localization of calumenin in hippocampal cell cultures, it was important to investigate whether the developmental changes of calumenin levels in culture were similar to those observed in vivo. As shown in Fig. 6A, a continuous decrease in calumenin levels was observed over time ranging from DIV 3 to DIV 11. This is in accordance with our biochemical results from brain tissue and shows an overall tendency for a decrease of calumenin expression over time.

Subsequently cultured rat hippocampal cells at DIV 12–14 were co-stained with antibodies against calumenin and different subcellular markers. Control experiments indicated that the anti-calumenin antibody pre-adsorbed by over-expressed calumenin (Fig. 6B, left), in contrast to mock-adsorbed antibody (Fig. 6B, right, arrows), did not detect its target anymore. This again demonstrated the specificity of the calumenin signal obtained.

As depicted in Fig. 6C–E, the highest calumenin immunoreactivity (shown in green) was observed in the somatic region of the cells. To investigate whether the previously reported association of calumenin with the ER and Golgi was analyzed. Prominent levels of co-localization of calumenin with markers for the ER and Golgi was analyzed. Prominent levels of co-localization of calumenin with markers for the ER and Golgi was analyzed. 

DISCUSSION

In the present study the expression of the Ca$^{2+}$-binding protein calumenin in rodent CNS was analyzed. Protein levels of calumenin in brains of both P0 and adult mice are comparable with levels in heart, lung, and stomach. Calumenin mRNA transcript expression occurs throughout the CNS of P0 mice and decreases with age to reach a low expression level in adult mice. A similar decline in calumenin expression can also be observed in hippocampal cell cultures in vitro over time. In accordance with our in situ hybridization data, immunohistochemical stainings of P0 mice revealed a moderate expression of calumenin throughout the brain, with the highest signal intensity in cells situated in the area around the lateral ventricle - in germinal zones containing multipotent neural stem cells and radial glia (Hatten, 1999; Parmavelas, 2000; Wichterle et al., 2001). Cells in these zones represent migrating neurons, which are born in neocortical ventricular zone and probably give rise to the principal pyramidal neurons of the neocortex (Sidman and Rakic, 1973; Parmavelas, 2000; Walsh and Goffinet, 2000). It has been well documented that intracellular calcium and calcium-binding proteins play a key role in many processes during the development of the central nervous system, including cell division, neuronal migration, and cell movement (Kwong et al., 2000; Konur and Ghosh, 2005; Berglund and Augustine, 2008). Notably, calumenin was described in functional association with the cytoskeleton in HeLa cells and fibroblasts (Ostergaard et al., 2006; Bull et al., 2010). Cytoskeleton on the other hand plays an essential role in neuronal...
movements during migration (Schaar and McConnell, 2005). Taken together, these data indicate a possible im-
portant role of calumenin in migration and differentiation of neurons during the CNS development.
Upon immunohistochemical staining of adult mouse brain, distinct populations of cells expressing calumenin could be detected. In cerebellum, the majority of cells stained for calumenin were situated in the PCL, and they most probably represent Bergmann glia cells. Calcium signaling plays a key role in regulating structural and functional interactions between Bergmann glia and Purkinje cell synapses (Metea and Newman, 2006). Calumenin could thus be one of the proteins involved in Ca$^{2+}$ homeostasis of Bergmann glia cells. In dentate gyrus of hippocampus, individual NeuN-positive cells at the border between granule cell layer and molecular layer, as well as in subgranular zone, also express calumenin and most probably represent interneurons. However, most of the calumenin-positive cells in dentate gyrus were not stained with NeuN, suggesting that they might represent glia. This was confirmed by co-staining of a population of cells in dentate gyrus with calumenin and astrocytic marker GFAP. Calcium signaling exerts important effects on glia homeostasis. In astrocytes, for example, calcium signaling is not restricted to single cells but can cross cell borders via gap junctions, resulting in intracellular Ca$^{2+}$ waves traveling from one glia cell to the next, or in the induction of Ca$^{2+}$ responses in neurons (Verkhratsky, 1997). Calcium signaling might thus represent a form of glial excitability, enabling these cells to integrate extracellular signals, communicate with each other, and exchange information with neurons. Interestingly, most of NeuN negative cells expressing calumenin were localized in the SGZ of dentate gyrus, one of two well established regions where adult neurogenesis takes place (Ming and Song, 2005). Moreover, some of these calumenin-positive cells were also expressing GFAP, the marker of progenitor cells and astrocytes, whereas some cells co-expressed calumenin with PSA-NCAM, the marker of differentiating and migrating progenitor cells (Kronenberg et al., 2003; von Bohlen und Halbach, 2007). Taken together, these immunohistochemical data strongly indicate that calumenin can be expressed by progenitor cells situated in SGZ of dentate gyrus and that it might play a role in early stages of adult neurogenesis.

Finally, on the subcellular level, calumenin was most abundant in membrane-enriched fractions obtained upon differential centrifugation of rat brain. Furthermore, a high immunoreactivity of calumenin was observed in the so-called neurogenic niche composed of the subgranular zone of the dentate gyrus and the subventricular zone, areas where proliferation and migration of neural stem cells take place (Kronenberg et al., 2003). The pattern of calumenin expression as well as its cellular localization is consistent with a role in early stages of adult neurogenesis (Tejedor et al., 2007). Additionally, calumenin could be detected in hippocampal interneurons (Beaulieu et al., 2009). A variety of synaptic markers (synaptophysin, SV2, PSD-95, and gephyrin) were used together with anti-calumenin antibody, to test whether calumenin could be found at synaptic sites. Using confocal microscopy, a significant co-localization of calumenin and the above mentioned synaptic markers could not be observed. However, to definitely exclude the presence of calumenin in those parts of the ER being in close proximity of synaptic sites (Ramírez and Couteel, 2011), a much higher resolution such as that of electron microscopy would be required.

Increased calcium concentration is observed not only under normal conditions in Ca$^{2+}$-storing organelles and in the extracellular space, but is also a hallmark of diverse neuropathological conditions, such as neurodegeneration (Mattson, 2007; Sammels et al., 2010; Supnet and Bezprozvanny, 2010; Surmeier et al., 2010). The role of various Ca$^{2+}$-binding proteins in neurodegeneration in both neuronal and glia cells has been well studied (Heizmann and Braun, 1992; Verkhratsky, 1997; Gómez-Tortosa et al., 2001; Nedergaard et al., 2010). Interestingly, calumenin has been demonstrated to associate with serum amyloid P component (Vorum et al., 2000), a member of the pentraxin family of plasma proteins, which is up-regulated in neurons of humans with Alzheimer disease (Yasojima, 2000). Future investigations should answer whether this interaction also takes place in the CNS and what the functional implications of such association are.

The present data establish a starting point for further analysis of the calcium-binding protein calumenin in the rodent brain. Due to its possible role in neuronal development and in Ca$^{2+}$-signaling between different cell types of the CNS, it might have an important function in health and disease.

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