Distribution of two molecules in rat cortical slices: KCC2, which plays an indirect role in the postsynaptic responses evoked through $GABA_A$ receptors, and δ-subunit-containing $GABA_A$ receptor, which mediates tonic currents.

Ph.D. thesis

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INTRODUCTION AND AIMS

The precisely regulated ionic homeostasis of the nerve cells is the basis of their electrical properties, and so, of the quality of their connections. Communication between neurons come off partially by synapses. The main inhibitory nerutransmitter in nervous system, the γ-aminobutiric acid (GABA) exerts its effect through the ionotropic $GABA_A$ receptors according to the intracellular Cl^- ion concentration, and through the metabotropic $GABA_B$ receptors. Kation-chloride cotransporters regulates neuronal Cl^- concentration and between them, K^+-Cl^- cotransporter KCC2 have been reported to be largely responsible for the hyperpolarizing GABAergic responses in mature nerve cells (Payne et al., 2003). Our previous experiments applying high resolution immunolocalization showed the relative lack of KCC2 in the axon initial segment compared to the soma of neocortical pyramidal cells (Szabadics et al., 2006). Low KCC2 levels in the plasma membrane of the axon initial segment of pyramidal cells can contribute to variable reversal potentials for perisomatic $GABA_A$ receptor mediated postsynaptic potentials (Szabadics et al., 2006; Khirug et al., 2008; Glickfeld et al., 2009; Woodruff et al., 2009). Potentially variable KCC2 concentrations in the dendritic region of pyramidal cells might explain some of the diverse results functionally testing the polarity of dendritic GABAergic responses (Alger and Nicoll, 1979; Andersen et al., 1980; Perkins and Wong, 1997; Gulledge and Stuart, 2003; Glickfeld et al., 2009) but the distribution of KCC2 on dendrites of pyramidal cells remains to be established. We applied high-resolution pre-embedding immunolocalization to quantify KCC2 concentrations along dendritic, somatic and axonal regions of rat hippocampal principal cells.

Synaptic $GABA_A$ receptors mediate fast postsynaptic effect while extrasynaptic receptors act in tonic inhibition regulating cortical excitability (Semyanov et al., 2003) and information processing (Mitchell and Silver, 2003; Chadderton et al.,

2004). δ-subunit containing $GABA_A$ receptors ($GABA_A\delta$) has several properties that promotes its effective participation in tonic inhibition. Examining the extrasynaptic effects of neurogliaform cells we focused on delta subunit containing $GABA_A$ receptors that act through tonic inhibition. For the localization we used immunofluorescent method on the neocortex.

METHODS

Preparation of tissue for immunocytochemistry.

All procedures were performed with the approval of the University of Szeged. Adult Wistar rats (n = 12) were deeply anesthetized by the intraperitoneal injection of tribromoethanol (10 ml/kg). Three rats were perfused for fluorescent labeling of KCC2 first with physiological saline for 1 minute, then with ice-cold fixatives, containing 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid made up in 0.1 M phosphate buffer (PB) for 15-30 minutes and five rats with this fixatives without glutaraldehyde for 10 minutes. The brains were immediately removed, and kept in 0.1 M PB. Four animals were perfused for electron microscopy through the aorta with 0.9% saline for 1 minute, then three of them with ice-cold fixative containing 4% paraformaldehyde and 4% acrolein in 0.1 M PB. In the remaining one, the perfusion was continued with Sloviter's solution of 0.1 M acetate buffer (pH 6) containing 2% paraformaldehyde and 0.1% glutaraldehyde for 2 minutes, then with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium borate buffer (pH 8.5) for 1 hour. After the perfusion with acrolein (n = 3) the brains were immediately removed from the skull, but the one fixed with Sloviter's solution was left in the skull for overnight at 4 ˚C and then removed. Horizontal and coronal sections (60 μm) were prepared with a vibratome (Leica VT 1000S), then collected in 0.1 M PB.

Immunofluorescence labelling.

Against the δ-subunits of $GABA_A$ receptors we kindly got polyclonal primary antibody from Sieghart (Center for Brain Research, Vienna, Austria). After the incubation with the primary antibody at 4°C for 72h, we used biotinylated secondary antibody and for the visualization of $GABA_A\delta$ receptor immunoreactions we treated the sections with ABC complex (Vector Laboratories) and used Alexa 488-conjugated tyramide signal amplification kits (Molecular Probes).

The sections for light microscopy were treated 30 minutes in 0.1M PB containing 1% hydrogen peroxide then washed several times in 0.1 M PB. All sections were incubated for 1 hour in blocking buffer (5% normal horse serum, 0.3% triton, 0.05% azide in 0.1 M PB) then placed into diluted rabbit anti-KCC2 at a final concentration of 2 μg/ml (Upstate, Lake Placid, NY 12946, 1:500) dissolved in blocking buffer, overnight at room temperature. After several washes in 0.1 M PB the immunoreactions were visualized with Cy3-conjugated donkey-anti-rabbit antibody (Jackson ImmunoResearch, Suffolk, UK, 1:250). Images were taken by an upright light microscope (BX60, Olympus) using a 4x (NA = 0.13) objective and confocal laser scanning microscope (FV1000, Olympus) using a 20x (NA = 0.85) objective.

Adult Wistar rats (n = 5) and $GABA_A$-receptor δ-subunit -/- mice (n = 2) were perfused with fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffer (PB, pH 7.3) for 10min in deep anaesthesia. For immunoreactions concerning δ-subunits of $GABA_A$ receptors, 60-μm-thick coronal sections were incubated in citrate buffer containing 10mM citric acid and 0.05% Tween20 (Sigma) in distilled water (pH6.0) at 95–100°C for 10min. After cooling to room temperature, sections were blocked with normal horse serum (NHS, 10%) in Tris-buffered saline (TBS, pH7.4) for 1h and incubated

with primary rabbit polyclonal antibody (1:500) diluted in TBS containing 2%NHS and 0.1% TritonX-100 for 72h at 4°C. Washes were done between steps with TBS containing 0.05% Tween 20 until incubation in a cocktail containing biotinylated donkey anti-rabbit secondary antibody (1:250; Jackson Immunoresearch), and thereafter with TBS only. Sections were treated with ABC complex dissolved in TBS (1:100; Vector Laboratories) and $GABA_{A\delta}$-receptor immunoreactions were visualized using Alexa 488-conjugated tyramide signal amplification kits (Molecular Probes).

Colocalizations were performed afterwards with the following primary antibodies: mouse anti-α-actinin (1:40,000; Sigma), goat anti-parvalbumin (1:5,000; PGV-214, Swant), mouse anti-calbindin (1:100; C8666, Sigma), guinea pig anti-vasoactive intestinal peptide (1:200; T-5030, Peninsula Laboratories), mouse anti-calretinin (1:1,000; 6B3, Swant), rat anti-somatostatin (1:50; MAB354, Chemicon), and mouse anti-reelin (1:50,000; MAB5366, Chemicon). Primaries were diluted in the cocktail described above and were visualized using the following secondaries: Cy3-conjugated donkey anti-mouse (1:500; Jackson Immunoresearch), Cy3-conjugated donkey anti-rabbit (1:500; Jackson Immunoresearch), Cy3-conjugated donkey anti-rat (1:500; Jackson Immunoresearch), Cy5-conjugated donkey anti-guinea pig (1:500; Jackson Immunoresearch) and Alexa□350-conjugated donkey anti-goat (1:500; Molecular Probes). Finally, sections were mounted on slides in Vectashield (Vector Laboratories). Images were made using a light microscope (BX60, Olympus) with a ×5 objective or a confocal laser scanning microscope (IX81, Olympus) with a ×20 (numerical aperture, 0.75) or a ×40 (numerical aperture, 1.30) objective. Automated sequential acquisition of multiple channels was used. *Z*-stack images were made up from 3–9 images in 5–45μm depth of tissue.

Pre-embedding immunocytochemistry. The sections for electron microscopy were treated with 1% sodium borohydride in 0.1 M PB for 20 minutes, then washed several times in 0.1 M PB. To enhance antibody penetration, sections were freeze-thawed in liquid nitrogen. After extensive washes in Tris-buffered saline (TBS, pH 7.4), selected sections were blocked in 10% normal goat serum for 1 hour, then incubated overnight in 2% normal goat serum containing rabbit anti-KCC2 antibody (2 μg/ml, 1:500, Upstate) at room temperature. Following several washes in TBS, sections were incubated in secondary antibody (0.8 nm gold-conjugated goat anti-rabbit IgG, 1:50, BB International, Dundee, DD2 1 NH, UK) diluted in TBS containing 0.8% bovine serum albumin (Sigma, Saint Louis, Missouri 63103 USA) and 0.1% cold-water fish skin gelatine (Aurion Immunoresearch, Wageningen, The Netherlands) overnight, postfixed in 1% glutaraldehyde in TBS. The ultra small gold particles were silver enhanced using R-Gent SE-LM silver kits described by the manufacturer (Aurion). The sections were treated with 0.5% OsO_4 in 0.1 M PB for 20 minutes at 4˚C, contrasted in 1% uranyl acetate for 25 minutes, dehydrated in graded alcohol series and finally embedded in Durcupan (Fluka, Bluchs, Switzerland). When the application of the primary antibody was omitted from the protocol described above, no specific signal could be detected. The specificity of the anti-KCC2 antibody was extensively studied by the laboratory of origin (Williams *et al*., 1999) and was successfully used previously for detecting KCC2 expression pattern in different CNS areas by several laboratories (Rivera *et al*., 1999; Gulacsi *et al*., 2003). Hippocampal CA1 and DG areas were selected for further examination. Blocks containing these areas were re-embedded and 80 nm thick ultrathin sections were cut with an ultramicrotome (RMC MT-XL), and the ribbons of sections were collected on grids and examined with a Tecnai-12 (FEI) electron microscope. Images were taken by a CCD camera (MegaView III, Soft Imaging System).

Quantitative analysis of immunogold labelling.

Both fixation types for electron microscopic studies gave similar results, thus we pooled the data. Immunogold densities for cytoplasmic and membrane associated KCC2 labelling were established along the soma, dendritic shafts, dendritic spines and axon initial segments (AIS) of pyramidal cells in the following regions of the CA1 area: str. pyramidale ("0 μm"), proximal and distal part of the str. radiatum (0-100 μm and 230-300 μm from somatic layer, respectively) str. lacunosum moleculare (320-400 μm) and str. oriens (0-70 μm). Somatic, dendritic shaft and AIS plasma membrane associated KCC2 densities were determined along DG granule cells by dividing stratum moleculare for a proximal and a distal part (0-40 μm and 140-180 μm from somatic layer, respectively, Table 1). Pyramidal and granule cells were identified by their spherical nucleus, dendritic shafts receiving no asymmetrical synapses and by the occurrence of dendritic spines. Profiles were identified as spines when receiving asymmetrical synapses and containing no mitochondria. Electron micrographs were taken in a depth of < 2 μm from the tissue surface, and special care was taken to investigate the immunoparticle densities in the same depth. First, we calculated the nonspecific labelling density over pyramidal nuclei (in gold/μm^2) and along nuclear plasma membrane (in gold/μm) which should not contain any KCC2. We measured the distances of gold immunoparticles from the plasma membrane. The values showed a Gaussian distribution with a peak location of 18.8 nm and s.d. of 11.1 nm so we considered gold particles within 41 nm inside the cells attaching to the plasma membrane (±2 x s.d. around the mean), resulting in an effective membrane width of 41 nm. Membrane associated KCC2 densities were determined in immunoparticle per effective membrane area (gold/μm^2) establishing a comparision with cytoplasmic immunogold densities and in immunoparticle per cut membrane length (gold/μm) for comparision of membrane associated KCC2 labeling using Reconstruct software (Synapse Web, Kristen M. Harris, PI,

http://synapses.clm.utexas.edu/). Data are given as mean ± s.d. All densities were compared to the non-specific background labelling (cytoplasmic to cytoplasmic and membrane associated to membrane associated values) with a paired *t*-test. Then, significantly different sets of data were further compared after substraction of background labelling in each animal with normalization of the means to corresponding somatic density. Paired comparisons were done using ANOVA statistical analysis. In order to circumvent statistical effects of homogeneity, we inspected F values with Brown-Forsythe and Welch procedures showing significant values ($P < 0.001$) in each case and then analyzed our data with Games-Howell post hoc test which does not rely on the assumption of equal variances.

RESULTS AND DISCUSSION

I. Distribution of KCC2 along the axo-somato-dendritic axis of hippocampal principal cells: CA1 pyramidal cells and DG granule cells.

We applied high-resolution pre-embedding immunolocalization to quantify KCC2 concentrations along dendritic, somatic and axonal regions of rat hippocampal principal cells. Confirming our results on neocortical pyramidal cells, membranes of AIS of CA1 pyramidal cells and dentate granule cells contained 6.4 ± 11.9% and 6.6 ± 14.1% of somatic KCC2 concentrations, respectively. Concentrations of KCC2 in basal dendritic shafts of stratum (str.) oriens were similar to somatic levels (109.2 ± 48.8%). Along apical dendritic shafts of CA1 pyramidal cells, the concentration of KCC2 showed a complex profile: normalized to somatic levels, the density of KCC2 was 124.5 ± 15.7%, 79 ± 12.4% and 98.2 ± 33.5% in the proximal and distal part of str. radiatum and in str. lacunosum moleculare, respectively. Dendritic spines of CA1 receiving excitatory inputs contained 39.9 ± 8.5% of KCC2 concentration measured in

shafts of the same dendritic segments targeted by GABAergic inputs. Dendrites of dentate granule cells, the other glutamatergic cell type in hippocampus, showed higher KCC2 concentration compared with the soma (148.9 ± 54%), but no concentration gradient was detected between proximal and distal dendrites. In conclusion, the density of KCC2 in hippocampal principal cells increases along the axo-somato-dendritic axis with cell type-specific distribution profiles within the dendritic tree.

The axo-somato-dendritic increase in KCC2 density in both principal cell types of the hippocampus suggest that relatively distal dendritic responses through $GABA_A$ receptors are likely to be hyperpolarizing (Glickfeld et al., 2009), possibly with a more negative reversal potential than proximal somatodendritic synapses. Indeed, increasing KCC2 concentrations and/or KCC2 versus GABAergic input ratios in the apical dendrite described here could contribute to the apparent reversal potential difference measured for somatic and dendritic GABAergic signals (Gulledge and Stuart, 2003; Szabadics et al., 2006; Khirug et al., 2008; Woodruff et al., 2009), but see (Glickfeld et al., 2009). Furthermore, the higher Cl^- extrusion capacity relative to symmetrical synapses in distal apical or thin basal dendrites might help in keeping intracellular Cl^- homeostasis and the polarity of $GABA_A$ receptor mediated response polarity during sustained GABAergic bombardment which can be related to normal cortical function or to pathological conditions such as trauma, epilepsy, oxidative stress, or ischemia known to downregulate the expression of KCC2 (Nabekura et al., 2002; Payne et al., 2003; Pond et al., 2006; Wake et al., 2007; Papp et al., 2008).

II. Distribution of δ subunit containing $GABA_A$ receptors

For the localization of δ subunit containing $GABA_A$ receptors we used immunofluorescent method on the neocortex and hippocampus. In addition to a robust neuropil labeling in supragranular layers of neocortex presumably due to

dendrites of pyramidal cells, a subset of interneurons was strongly positive for the $GABA_A\delta$. The identity of strongly $GABA_A\delta$ immunopositive interneurons was initially tested by multiple immunoreactions showing that α-actinin2, known to be expressed by electrophysiologically identified neocortical neurogliaform cells, were present in 65 ± 12% of $GABA_A\delta$ receptor containing cells. However, no overlap was found with interneuron markers such as parvalbumin, somatostatin, calbindin, calretinin and vasoactive intestinal polypeptide. In addition, immunocytochemical labeling patterns in the hippocampus were in line with earlier electrophysiological data showing relatively large tonic inhibition in hippocampal granule cells and molecular layer interneurons in the dentate gyrus and weaker currents in pyramidal cells of the hippocampus. In the CA1 and CA3 regions, a subset of interneurons at the border of stratum radiatum and lacunosum moleculare and another population of interneurons close to stratum pyramidale show strong immunolabeling for $GABA_A\delta$ receptors. These interneurons presumably correspond to hippocampal neurogliafrom cells and Ivy cells, respectively. Neurosteroids might shift the balance among the sources of ambient GABA by lowering the contribution of neurogliaform cells with a selective increase in tonic inhibition through $GABA_A\delta$ receptors. Varying neurosteroid concentrations during the ovarian cycle and pregnancy and as a result of stress (Stell et al., 2003; Glykys and Mody, 2007a) are expected to modulate the action of neurogliaform cells on network hyperpolarization and in suppressing communication in the local circuit acting on axons of resident neurons or terminals of long-range projections at their arrival (Olah et al., 2009).

PUBLICATIONS

Rita Báldi, Csaba Varga, Gábor Tamás: Differential distribution of KCC2 along the axo-somato-dendritic axis of hippocampal principal cells. Eur J Neurosci. 2010. Oct:32(8):1319-25

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CONFERENCE POSTERS

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Rita Báldi, Csaba Varga, Gábor Tamás; Differential distribution of KCC2 along the axo-somato-dendritic axis of CA1 pyramidal cells; MITT konferencia, Budapest, Magyarország, 2009

Rita Báldi, Csaba Varga, Gábor Tamás; Differential distribution of KCC2 along apical dendrites of CA1 pyramidal cells; 6th FENS Forum of European Neuroscience, Genf, Svájc, 2008

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