Contribution of a glial glutamate transporter to GABA synthesis in the retina

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Received 28 May 2004; accepted 22 June 2004

Neuronal glutamate transporters have been shown to play a role in GABA synthesis by enhancing glutamate uptake. In the present study, we have examined whether a glial glutamate transporter, GLAST, has a role in GABA synthesis in the mammalian retina. We found that the retinal GABA level was about two-fold higher in the GLAST$^{-/-}$ mouse retina compared to that in the wild type. Endogenous glutamate level was also increased about 2-fold in the mutant. Therefore, loss of GLAST results in a higher retinal GABA level, probably due to increased availability of its precursor, glutamate. An increase in GABAergic activity can be expected to affect trigger features such as directional selective response of neurons in the GLAST$^{-/-}$ mouse retina. *NeuroReport* 15:1895–1898

Key words: GABA; GLAST; Glutamine; Glutamate transporter; Knockout mice; Müller cell; Retina

INTRODUCTION

Glutamate plays a dual role in synaptic transmission. At excitatory synapses, it functions as a neurotransmitter to convey signal to post-synaptic neurons whereas at inhibitory synapses it serves as the substrate for the synthesis of GABA, a major inhibitory neurotransmitter. In the CNS, extracellular glutamate levels are regulated by high-affinity glutamate transporters that are found on both glial cells and neurons [1]. Based on DNA sequence, pharmacology, and channel properties, at least five subtypes of sodium-dependent, glutamate transporters have been identified and characterized: GLAST-1 (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5 [2]. EAAC1 is localized to neurons whereas GLT1 and GLAST are the main glutamate transporters in glial cells. EAAT4 is found predominantly in the cerebellum while EAAT5 appears to be restricted to the retina [2].

Plasma membrane glutamate transporters maintain extracellular glutamate at a low level, and thereby protect neurons from excitoxicity, and also ensure a high signal-to-noise ratio for glutamatergic neurotransmission [1]. In addition, neuronal glutamate transporters have been shown to play a role in GABA synthesis. For example, the glutamate transporter EAAC1 has been shown to be present on the inhibitory terminals of GABAergic neurons where it might enhance glutamate uptake and thus, increase the glutamate pool available for GABA synthesis [3–5]. More direct evidence is provided by experiments in which suppression of EAAC1 expression in hippocampal slices using antisense oligonucleotides was found to decrease GABA levels by 50%, and to reduce new GABA synthesis [6]. It was shown recently that glutamate transporters regulate the vesicle pool of GABA in hippocampal CA1 interneurons, and that reduced glutamate uptake diminished evoked IPSCs and mIPSCs without affecting post-synaptic receptors [7]. These observations suggest that the neuronal glutamate transporter, EAAC1, contributes to GABA formation. Whether the other EAATs have a similar role in GABA synthesis has not been studied.

Electrophysiological studies suggest that GLAST is the primary regulator of extracellular glutamate level in the retina [8], while EAAC1, GLT-1 and EAAT5 appear either to influence synaptic glutamate level or to regulate the amount of glutamate that escapes the synapse [9]. Therefore, one might expect that extracellular glutamate level might be elevated in the retinas of GLAST $^{-/-}$ mice. As a result, there may be an increase in GABA content in neurons due to increased precursor availability [10]. Because GABAergic neurons are important for the establishment of directional sensitivity in the mammalian retina [11,12], we wanted to investigate whether GABA level is altered in the GLAST$^{-/-}$ mouse retina.

MATERIALS AND METHODS

Animals: GLAST knockout (GLAST$^{-/-}$) mice were obtained by mating GLAST$^{+/-}$ (heterozygote) mice and identified by PCR of tail DNA using GLAST-specific oligonucleotide primers [8]. The generation and characterization of the GLAST knockout mice has been described

earlier [13]. C57B6/6J mice were purchased from Harlan Sprague Dawley, Indianapolis, IN, USA. All animals were maintained in clear plastic cages with standard light cycles (12:12 h light:dark) and handled according to approved protocols. Experimental procedures were designed to conform to the NIH guidelines for the use of animals in biomedical research.

GABA immunocytochemistry: Mouse retinas were fixed with 2.5% glutaraldehyde/1% formaldehyde in 0.1 M pH 7.4 phosphate buffer supplemented with 3% sucrose and 1 mM MgSO4, dehydrated in graded methanols, and embedded in epoxy resin [14]. Serial thin 250 nm sections were fully etched in sodium methoxide and probed for GABA, L-glutamate and L-glutamine using anti-hapten IgGs described previously [15,16]. Signals were visualized with calibrated silver-intensified 1 nm immunogold secondary IgGs, channels registered with <200 nm precision with PCI Geomatica (Richmond Hill, Canada) remote sensing code (see [14] for details) and analyzed with in-house multivariate analysis code (CellKit V1.0.6) developed under IDL V from RSI (Boulder, CO). Signal histograms were calibrated as intracellular concentration by remapping pixel values to known concentrations in ovalbumin-lysine-amino acid standard stacks similar to those fabricated in previous studies [17].

Amino acid analysis by HPLC: The concentration of amino acids in the retina was assayed by HPLC. Tissue samples ($\sim$10 mg) were homogenized in 140 µl 0.1 M TCA (0.1 M TCA containing 0.02 M CH_3COONa, 1 mM EDTA and 9.0% methanol). α-Aminobutyric acid (250 pmol/µl) was included in the homogenizing solution to serve as an internal standard. To obtain the fluorescent derivatives, 10 µl samples were added to 70 µl borate buffer (part of kit (AccQ-Tag Chemistry Package, WAT052875) supplied by Waters Corporation Milford, MA) and 20 µl 6-aminoquinol-N-hydroxysuccinimidyl carbamate solution (part of kit AccQ-Tag Chemistry Package, WAT052875 supplied by Waters Corporation, Milford, MA, USA) and 20 µl 6-aminoquinol-N-hydroxysuccinimidyl carbamate solution (part of kit AccQ-Tag Chemistry Package, WAT052875). After heating the mixture for 10 min at 37°C, 10 µl of the labeled sample was injected into the HPLC system consisting of a Waters 712 autosampler, two 510 HPLC pumps, a column heater (37°C) and a Waters 474 scanning fluorescence detector (excitation wavelength 250 nm, emission wavelength 395 nm). Separation of the amino acids was accomplished using a Waters amino acid HPLC column with buffer A: 19% sodium acetate, 7% phosphoric acid, 2% triethylamine, 72% water; and buffer B: 60% acetonitrile, using a specific gradient profile. HPLC control and data acquisition was managed by Millennium 32 software. Using this HPLC solvent system, the amino acids elute in the following order: cysteine, homocysteine, aspartic acid, serine, glutamate, glycine, glutamine, histidine, taurine, arginine, threonine, alanine, proline, GABA, cystine, tyrosine, valine, methionine, lysine, isoleucine, leucine, and phenlyalanine. Calibration was obtained by running daily calibration curves, consisting of known concentrations of each amino acid (10 pmol/µl–100 pmol/µl) to which the internal standard (GABA, 250 pmol/µl) was added. The peak heights of each amino acid were compared to that of the internal standard. The resulting calibration curve was used to determine the concentration of the samples being tested.

RESULTS

Immunohistochemical localization of GABA, glutamate and glutamine: Our initial studies focused on localization of GABA and L-glutamate in the $GLAST^{-/-}$ mouse retina using established immunocytochemical methods [16]. For comparison, we also carried out a similar study in wild type mice. Results of the experiment are presented in Fig. 1a,b. In the $GLAST^{-/-}$ mouse retina, GABA-immunoreactive cell bodies were found in the inner nuclear layer (INL) and the ganglion cell layer (GCL) (Fig. 1b). In the INL, the majority of the labeled-cell bodies were located close to the INL/IPL (inner plexiform layer) border where GABAergic neurons are located [18]. In addition, GABAergic processes formed a dense network in the inner plexiform layer. A comparison with normal retina immunostained for GABA (Fig. 1a) suggested that neither the distribution nor the content of GABA was significantly altered in the retinas of $GLAST^{-/-}$ mice.

In the case of L-glutamate, immunostaining was strongest in rods, INL cell bodies (bipolar cells) and GCL cell bodies (ganglion cells). There was no change in the intensity or pattern of L-glutamate distribution in the retinas of $GLAST^{-/-}$ mice (Fig. 1a,b). The immunostaining of retinal cells we observed is consistent with the immunocytochemical localization of GABAergic and glutamatergic neurons reported in the mouse retina [19].

In addition to glutamate and GABA, we also examined glutamine distribution in normal and GLAST mouse retina (Fig. 1c,d). Glutamine was present at significant levels in cell bodies located in the middle of the INL and in processes in the inner and outer retina. Based on their location, the glutamine-immunoreactive, cell bodies are likely to belong to Müller cells.

Next, we used computational molecular phenotyping to estimate GABA levels in retinal cells. As described elsewhere, calibrated small molecular immunocytochemistry can accurately detect concentrations in the range 0.1–10 mM [16,17]. The specimens illustrated in Fig. 1a,b were segmented by pattern recognition and calibrated signal histograms were acquired by selectively masking Müller cells and GABAergic amacrine cells [15,17] from calibrated silver-intensified images of GABA immunoreactivity on 250 nm sections (Fig. 1e). Both wild type and $GLAST^{-/-}$ retinas were embedded as a stack in the same block face [15] and thus were probed concurrently. The integral under each histogram represents the concentration of GABA in that compartment alone (Fig. 1e). Both Müller cell- and amacrine cell contents were shifted to higher concentrations in the $GLAST^{-/-}$ retinas. The broad dispersion in amacrine cell signals represents the diversity of GABA concentrations across GABAergic amacrine cells. The GABA content of some GABAergic amacrine cells in $GLAST^{-/-}$ retinas was clearly shifted outside the upper range of our detection (saturation of silver intensification). The retinal fraction of Müller cells, as estimated by masking [14,16], was 0.23 for $GLAST^{-/-}$ and 0.22 for wild type retinas. The corresponding fractions of GABAergic amacrine cells were 0.14 and 0.11. Scaling for fractional mass, imaging suggested that the

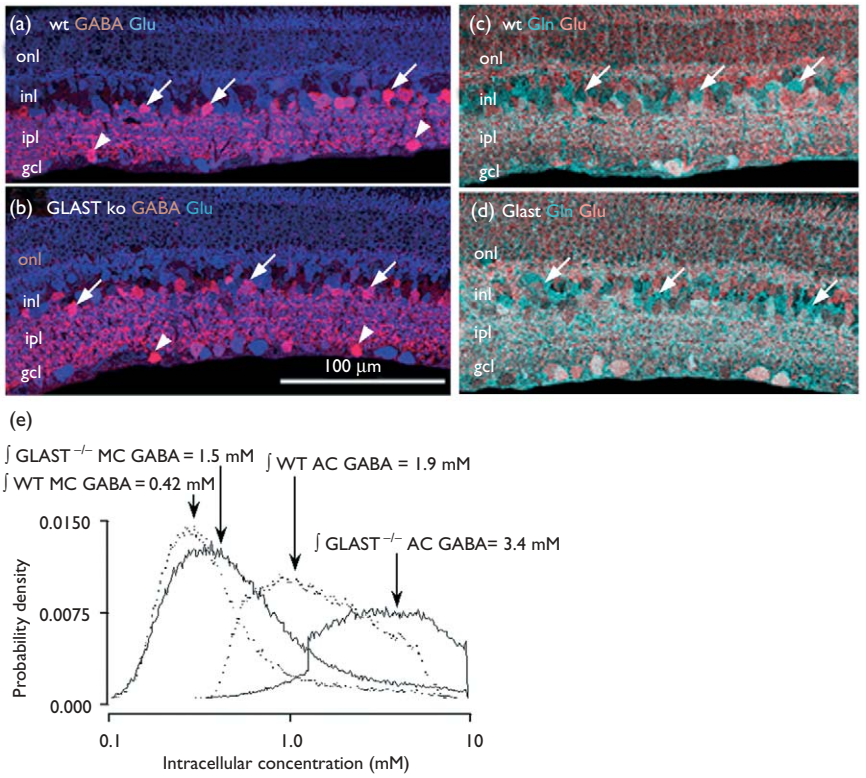

Fig. 1. Immunocytochemical localization of GABA, glutamate and glutamine. Isolated retinas were fixed in an aldehyde mixture and processed for immunocytochemistry as described elsewhere [14]. Retinal sections (250 nm) were processed concurrently by probing with anti-GABA and anti-L-glutamate (1:10 000 dilution), and visualized with silver intensified immunogold. The micrographs show endogenous GABA and glutamate in wild type (**a**) and GLAST $^{-/-}$ mice (**b**). The distribution and content of GABA (red) and glutamate (blue) were similar in wild type and GLAST $^{-/-}$ mice. Arrows point to GABAergic neurons in the inl and arrow heads show GABAergic neurons in the gcl. (**c,d**) Immunocychemical localization of glutamate (red) and glutamine (cyan) in wild type and GLAST $^{-/-}$ mouse retina, respectively. Arrows point to glutamine-labeled cell bodies in the inl. The distribution of glutamate and glutamine are similar in wild type and GLAST $^{-/-}$ mice. (**e**) Pairs of quantitative probability density histograms for endogenous GABA signals in Müller cells (MC) and amacrine cells (AC) from GLAST$^{-/-}$ (solid lines) and wild type (WT) (dashed lines) mice, detected concurrently with quantitative, calibrated immunocytochemistry. All samples were 250 nm sections, probed simultaneously and represent the intracellular signals from Müller cells and amacrine cells in GLAST$^{-/-}$ and wild type mice. GABA signals were higher in the GLAST$^{-/-}$ Müller cells and amacrine cells. Bar=100 μm.

mean retinal content of GABA in the GLAST$^{-/-}$ retina was 2.7-fold greater than in wild type retinas.

Amino acid levels: A limitation of the immunocytochemical method is that it is not sensitive enough to detect small (<2-fold) changes in amino acid levels in the tissue. Because the immunocytochemical data suggested that there was a difference in GABA content between the mutant and wild type retina, we decided to determine endogenous GABA and L-glutamate levels by HPLC. Table 1 presents the endogenous levels of GABA and glutamate in retinas from GLAST$^{-/-}$ and wild type mice. GABA level was about 2-fold higher in the mutant compared to the wild type. Interestingly, endogenous glutamate level was also elevated about 2-fold in the mutant. These data suggest that the retinas in the GLAST$^{-/-}$ mice have higher endogenous levels of GABA and glutamate. The ratio of GABA:Glutamate, however, is about the same for the mutant (0.34) and wild type mice (0.35).

Because glutamate is also the precursor for glutamine synthesis in Müller cells, we investigated the relationship between glutamine level and glutamate content in retinas from GLAST$^{-/-}$ mice. Surprisingly, retinal glutamine levels were nearly the same in the mutant and wild type (Table 1). A comparison of glutamine: glutamate ratio, however, shows that more glutamate is converted to glutamine in the wild type (0.77) than in the mutant retina (0.37). This result suggests that there is decreased glutamate availability for glutamine synthesis in the mutant, probably as consequence of loss of GLAST, the major glutamate transporter in Müller cells.

Table 1. Endogenous levels of GABA, glutamate and glutamine in GLAST$^{-/-}$ and wild type mouse retina.

Amino acid	GLAST$^{-/-}$	Wild type
GABA	56.0 ± 4.4	26.9 ± 2.8
L-Glutamic acid	166.2 ± 9.9	76.4 ± 5.1
L-Glutamine	62.6 ± 7.6	59.3 ± 5.6
GABA/Glu	0.34	0.35
Gln/Glu	0.37	0.77

Amino acids are expressed as pmol/μg protein. Values are mean ± s.e.m. for 5–7 separate determinations each using four retinas. GABA, γ-Aminobutyric acid; Glu, Glutamic acid; Gln, Glutamine.

DISCUSSION

The present studies show that loss of GLAST leads to an increase in GABA and glutamate content of the mouse retina. The amino acid levels found in retinas from wild type mice are in general agreement with previous data from Cohen *et al.* [20]. For GABA, glutamate and glutamine, our respective mean ($\pm$ s.e.m.) values are (in pmol/μg protein) 26.9 ± 4.4, 76.4 ± 5.1 and 59.3 ± 5.6, whereas Cohen *et al.* [20] reported values of 16.7 ± 0.7, 45.7 ± 1.3 and 15.2 ± 0.8. The consistently higher levels we found are likely to be due to the higher sensitivity of the HPLC method. Interestingly, the GABA/glutamate ratio was similar in the two cases.

Our observation differs from the findings of Seputsky *et al.* [6] who reported that antisense-blocking of EAAC1 resulted in a decrease in GABA content of hippocampal neurons. The discrepancy is probably related to the presence of EAAC1 in the inhibitory terminals of GABAergic neurons where it is likely to be involved in glutamate transport [3–5]. Suppression of EAAC1 would decrease glutamate transport and hence, reduce the glutamate pool available for GABA synthesis. In the retina, loss of GLAST, the major glutamate transporter, can be expected to result in increased extracellular glutamate level [21]; thus, making more glutamate available for GABA synthesis. It is also possible that the observed changes in GABA and glutamate level are a reflection of retinal reorganization during development rather than due to alterations of glutamate flux and compartmentalization.

We also do not know the reason for the higher endogenous level of retinal glutamate in $GLAST^{-/-}$ mice. The increase cannot be attributed to strain differences because both the mutant and the wild type have the same C57/B6 background. It is possible that, in the absence of GLAST, there is decreased L-glutamate uptake by Müller cells, and more glutamate is available for re-uptake by retinal neurons. The doubling of GABA level is likely to be due to the 2-fold change in the content of L-glutamate, its precursor.

What are the consequences of the changes in GABA and L-glutamate levels on retinal function? Because both GABA and glutamate are elevated in the mutant, there might be no net change in the inhibitory or excitatory inputs in the retinas of $GLAST^{-/-}$ mice. However, if there is an increase in the GABAergic activity, the retinal IPSCs and mIPSCs would also increase, which in turn might affect many retinal processes such as center-surround balance, contrast gain control, and direction selective response of neurons in the $GLAST^{-/-}$ mouse retina. Further experiments using electrophysiological and optic imaging techniques would be needed to test this idea [22].

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Acknowledgements: This work was supported by NIH grants, R01 EY-12135 (VS) and R01 EY-02576 (RM), and by unrestricted funds from Research to Prevent Blindness, Inc.