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BRAIN RESEARCH

Short Communication

Role of glutamate decarboxylase (GAD) isoform, GAD_{65}, in GABA synthesis and transport into synaptic vesicles—Evidence from GAD_{65}-knockout mice studies

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ABSTRACT

In GAD_{65}-knockout mice, lack of GAD_{65} expression was confirmed. The expression level of vesicular GABA transporter (VGAT) was upregulated, and no change in the synaptic vesicles (SV)-associated GAD_{67} was found. GAD_{65}(–/–) SV transported cytosolic GABA much more efficiently than that of the wild type, further supporting our model that there is a structural and functional coupling between GABA synthesis and packaging into SV. Both full-length and truncated forms of GAD_{65} could bind to GABAergic SV, indicating the N-terminus is not required for the anchoring of GAD_{65} to SV. Although both GAD_{65}(–/–) SV reconstituted with either GAD_{65} or GAD_{67} could synthesize GABA from [^{3}H] glutamate and transport this newly synthesized GABA into SV, the combined evidence suggests that GAD_{65} plays a major role in GABA transmission in normal physiological condition. However, GAD_{67} could serve this role under some pathological conditions.

GABA is well documented as a major neurotransmitter in the mammalian central nervous systems (Kravitz, 1967; Roberts and Kuriyama, 1968; Roberts, 1976; Olsen, 1991). In the brain, GABA is produced from L-glutamic acid, which is catalyzed by L-glutamic acid decarboxylase (GAD; EC 4.1.1.15), the rate-limiting enzyme (Roberts and Frankel, 1951). In vertebrates, there are two well-characterized GAD isoforms in the human brain, namely GAD_{65} and GAD_{67} (referring to GAD with a molecular mass of 65 kDa and 67 kDa, respectively), which are the products of two separate genes (Erlander et al., 1991; Bu et al., 1992; Lernmark, 1996; Bosma et al., 1999). Previously, we have shown the functional coupling between the GABA synthesis and its transport into synaptic vesicles (SV) using GABAergic SV (Jin et al., 2003). We also proposed a model that GAD_{65} forms a complex with vesicular GABA transporter (VGAT), which ensures an efficient coupling between GABA synthesis and packaging into the SV and partially blocks the vesicular transport of pre-existing GABA. GAD_{65}-knockout mice, which lack GAD_{65}, show susceptibility to seizure, indicating a weakness of GABA transmission (Asada et al., 1996). To test our hypothesis that GABA synthesis by GAD_{65} and packaging into the SV are efficiently coupled (Jin et al.,

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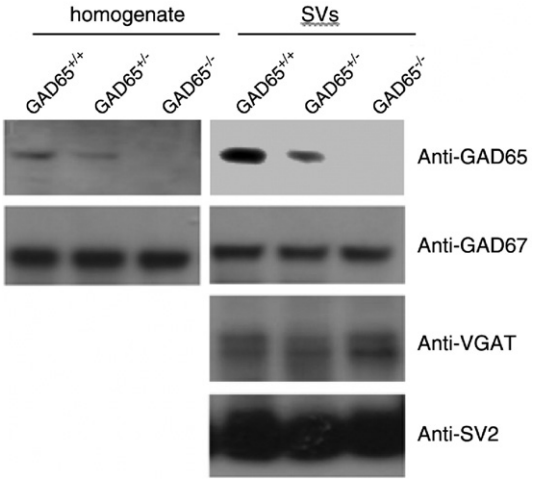

Fig. 1 – Immunoblotting analysis of GAD_{65}, GAD_{67} and VGAT. Brain homogenate and SV were prepared from GAD_{65}(+/+), GAD_{65}(+/-) and GAD_{65}(–/–). Equal amount of protein was loaded for each sample and detected using specific antibodies against GAD_{65}, GAD_{67} and VGAT as indicated. The protein SV_2 was used to show the equal loading.

2003), GAD_{65}-knockout mice were used in this study. The information obtained from this study will address the controversy in the literature, namely whether GAD_{65} or GAD_{67} is more involved in synthesis of GABA for synaptic vesicle packaging.

SV were prepared from whole-mouse brain (C57BL/6) at 4 °C as described (www.els.net) with some modification. Briefly, frozen mouse brains [wild type (+/+), heterozygous (+/–) and GAD_{65} knockout (–/–)] were homogenized in 0.3 M sucrose buffer (10 ml/g brain) and centrifuged at 15,000×*g* for 30 min. The resultant supernatant liquid (S_1) was collected. The pellet (P_1) was lysed by rapid resuspension in 10 volume of water; 130 mM potassium chloride was then added after 1 min. The suspension was centrifuged at 15,000×*g* again for 30 min. The resulting pellet (P_2) was discarded. The resulting supernatant solution (S_2) was combined with S_1 and was further centrifuged at 200,000×*g* for 2 h. The resulting pellet (P_3) was suspended in 10 vol of standard GAD buffer containing 50 mM potassium phosphate, 1 mM 2-aminoethylisothiouronium bromide (AET), and 0.2 mM pyridoxal 5′-phosphate (Sigma) at pH 7.2 and referred to as crude SV.

The anti-VGAT IgG-coupled protein G beads (Sigma) were used to purify GABAergic-specific SV from the crude SV preparation as described above. Briefly, specific anti-VGAT IgG was raised against synthetic peptide corresponding to amino acid residues 75–87 of VGAT (Synpep). Anti-VGAT IgG was first purified from anti-VGAT sera using protein G sepharose column. An aliquot of anti-VGAT IgG was added to protein G beads to give a ratio of 4 ml of IgG/g of bead. The mixture was incubated at 20–25 °C for 24 h, The beads were then extensively washed with standard GAD buffer. Anti-VGAT-coupled beads were incubated with crude SV preparation for 12–16 h at 4 °C, followed by an extensive wash with standard GAD buffer. The resulting bead complex was regarded as GABAergic-specific SV.

GABAergic SV prepared from GAD_{65}-knockout mice were incubated with full-length GAD_{65}, or full-length GAD_{67}, which were prepared as described previously (Davis et al, 2000) overnight at 4 °C. To test if N-terminus is involved in membrane anchorage of GAD_{65}, we also incubated GABAergic SV with the truncated form of GAD_{65} with first 69 residues deleted, because we previously showed that GAD_{65} could be cleaved to release a stable truncated form which lacks amino acids 1–69 from the N-terminus. The SV were then extensively washed with standard GAD buffer after incubation. The reconstituted SV were used for immunoblotting analysis (anti-GAD_{65} antibody was used) and GABA uptake assay.

GAD activity was measured by using a radiometric method as described (Wu et al., 1986). Immunoblotting tests were conducted as described (Hsu et al, 2000). The protein samples were first separated by SDS-PAGE using pre-casting BisTris–gel (Invitrogen) and then transferred onto a nitrocellulose membrane. The blot was detected by specific primary antibodies including anti-GAD_{65}, anti-GAD_{67}, anti-VGAT and anti-SV2 antibodies, and HRP-conjugated secondary antibodies using ECL detection reagents (Amersham Pharmacia). Each immunoblotting experiment was performed twice.

GABA uptake assays were performed as described (Wang and Floor, 1994; Floor et al., 1995; Jin et al., 2003) with some modifications. A SV mixture containing a final concentration of SV beads (2 mg of protein per ml), pyruvate kinase (60 μg/ml) in buffer consisting of 9.5 mM KH_2PO_4, 40.5 mM K_2HPO_4, 8 mM KCl, 86.6 mM potassium gluconate, pH 7.4 (GPBS), was first incubated at 32 °C for 2 min, followed by the addition of the same volume of ATP mixture giving a final concentration of 2 mM ATP, 4.4 mM $MgSO_4$, 12 mM phospho(enol) pyruvate, 50 μM GABA, 2 mM Glu, and 0.1 μCi/μl [^{3}H]GABA. The mixture was further incubated at 32 °C, and an aliquot of 30 μl of the reaction mixture was removed and vacuum-filtered through nitrocellulose membrane at 5- and 10-min intervals. The membranes were washed twice with 5 ml of ice-cold GPBS buffer, and the radioactivity remaining on the membrane was measured in a scintillation counter. Vesicular uptake with newly synthesized GABA from glutamate was performed as described above, except that GABAergic GAD_{65}(–/–) SV reconstituted with GAD_{65} or GAD_{67} were used and [^{3}H]GABA was replaced by [^{3}H]Glu in the reaction mixture.

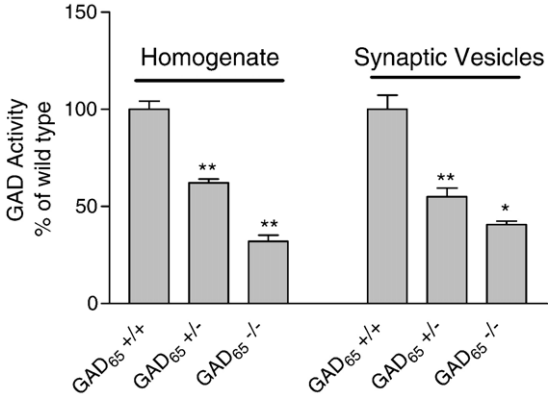

Fig. 2 – GAD activity in homogenate and SV from three groups was assayed as described. N=3. $^{**}P<0.01$, $^{*}P<0.05$ by Student's *t* test.