Glutamic acid decarboxylase 67 expression by a distinct population of mouse vestibular supporting cells

Elisa Tavazzani1, Simona Tritto1,2, Paolo Spaiardi1, Laura Botta2, Marco Manca1, Ivo Prigioni1, Sergio Masetto1 and Giancarlo Russo1*

1 Department of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy
2 Laboratory of Neurophysiology, Brain Connectivity Center, C. Mondino National Neurological Institute, Pavia, Italy

*Correspondence:
Giancarlo Russo, Department of Brain and Behavioral Sciences, University of Pavia, Via Forlanini 6, Pavia 27100, Italy
e-mail: gianca@unipv.it
†These authors have contributed equally to this work.

INTRODUCTION

Glutamic acid decarboxylase (GAD) catalyzes the conversion of L-glutamic acid to γ-aminobutyric acid (GABA). Glutamate decarboxylase exists as two major isoforms, termed GAD65 and GAD67, that are usually expressed in GABA-containing neurons in the central nervous system. GAD65 has been proposed to be associated with GABA exocytosis whereas GAD67 with GABA metabolism. In the present immunofluorescence study, we have investigated the presence of the two GAD isoforms in the semicircular canal cristae of wild type and GAD67-GFP knock-in mice. While no evidence for GAD65 expression was found, GAD67 was detected in a distinct population of peripherally-located supporting cells, but not in hair cells or in centrally-located supporting cells. GABA, on the other hand, was found in all supporting cells. The present result indicate that only a discrete population of supporting cells use GAD67 to synthesize GABA. This is the first report of a marker that allows to distinguish two populations of supporting cells in the vestibular epithelium. On the other hand, the lack of GABA and GAD enzymes in hair cells excludes its involvement in afferent transmission.

Keywords: GAD67-GFP, hair cells, supporting cells, crista ampullaris, vestibular

MATERIALS AND METHODS

Experiments were performed on transgenic C57BL/6 GAD67-GFP knock-in heterozygous mice which were generated by Tamamaki and Yanagawa (Tamamaki et al., 2003) and were generously supplied by Prof. G. Biella (Pavia). Mice were sacrificed from postnatal day (P) 10 to P26. No differences in the GAD67 expression were seen among these ages, and data were therefore pooled. All experimental procedures involving animals were approved by the Ministero Italiano della Salute, and comply with the European international laws on animal research. Prior to any surgery, deep anesthesia was obtained by means of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane).

Transgenic mice were obtained by crossing female wild-type C57BL/6 mice with male heterozygous GAD67-GFP mice.
Transgenic mice were sorted by two ways: (1) by examining the heads of P1-2 mice under a fluorescence lamp. GAD67-GFP knock-in mice exhibit a striking green fluorescence in the brain that can be visualized through the skull at this age; (2) by extracting DNA from mouse tails and carrying out the PCR. In the latter case, genomic DNA was extracted from mouse tail biopsies with the PureLink® Genomic DNA Midi Kit (Invitrogen, Italy). The extracted DNA was kept frozen at −80°C until use. PCR was performed on 2 µg DNA with the GoTaq® Flexi DNA Polymerase (Promega, Italy) and with specific primers for GAD67-GFP tagged mice (TR-1b 5′-GGCACAGCTCTCCCTTCTGTTTGC-3′; TR-3 5′-GCTCTCCTTTTCCGCTGACAG-3′; TRGFP-8 5′-CTGCTTGTGGCCATGATAGCG-3′). An initial denaturation at 94°C for 3 min was followed by 20 s at 96°C, 30 s at 68°C and 30 s at 72°C for 30 cycles. A final extension at 72°C for 10 min was performed. The molecular weight of the PCR products was compared to the DNA molecular weight marker VIII (Roche Molecular Biochemicals, Italy). The bands acquired with the Image Master VDS (Amersham Bioscience Europe, Germany) were at the expected size of 265 bp for GAD67 in wild type mice and of 265 bp and 564 bp for heterozygous GAD67-GFP mice (Figure 1).

**SLICE PREPARATION FOR CONFOCAL MICROSCOPY**

After 48 h, the fixed ampullae were embedded in 4% agar (Sigma-Aldrich, Italy) in extracellular solution, the agar blocks containing the ampullae were glued to the bottom of the Teflon plate of the vibroslicer chamber (Campden-Instrument, UK) filled with extracellular solution, and slices of the sensory epithelium of ~90 µm thickness were obtained.

Specimens were then mounted on slides for the confocal imaging. For the immunolabelling the specimens were washed with a 25% sucrose phosphate buffer solution (PBS), blocked for 60 min with 3% bovine serum albumin (Sigma Aldrich, Italy) in PBS and rinsed three times (5 min each) with PBS. Afterwards, the slides with the specimens were incubated overnight at 4°C with primary antibodies directed to calbindin (Calbindin D28K sc 7691 goat anti mouse, Santa Cruz Biotechnology, Italy) or to GAD65 (goat anti mouse 6113, Abcam, UK) or GABA antibody (guinea pig anti mouse ab17413 Abcam, UK), all diluted 1:100 in PBS. After three rinses in PBS (5 min each), the specimens treated with the primary antibodies were incubated overnight at 4°C with primary antibodies directed to calbindin (Calbindin D28K sc 7691 goat anti mouse, Santa Cruz Biotechnology, Italy) or to GAD65 (goat anti mouse 6113, Abcam, UK) or GABA antibody (guinea pig anti mouse ab17413 Abcam, UK), all diluted 1:100 in PBS. After three rinses in PBS (5 min each), the specimens treated with the primary antibodies were incubated (60 min at room temperature) with Alexa-fluor 633-secondary antibody conjugated (Life Technologies, Italy) or with Alexa fluor 594 secondary antibody (Life Technologies, Italy) at a dilution of 1:1000. The slides were then washed in PBS and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, Italy). Control experiments were performed simultaneously by omitting the primary antibody.

Fluorescence imaging was performed by a TCS SP5 II LEICA confocal microscopy system (Leica Microsystems, Italy) equipped with a LEICA DM IRBE inverted microscope. Images were acquired with 40X or 63X objectives and visualized by LAS AF Lite software (Leica Application Suite Advanced Fluorescence Lite version 2.6.0).
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FIGURE 2 | GAD67 expression in the mouse vertical cista. (A) Schematic representation showing a representative slice (gray) of the cista ampullaris. Dots represent hair bundles protruding from the cista surface. E.C.: eminentia cruciata; P.S.: planum semilunatum. (B,C,D) Photomicrographs of a superficial confocal section of the slice schematized in (A) showing the cell nuclei (blue; DAPI), the cellular expression of GAD67 (green; GFP), and the merged image, respectively. Light blue indicates co-localization of nuclei and GFP, which seems poor because GAD67 is expressed in the cytoplasm. Most cell bodies at this confocal level appear sectioned transversally. (E) Photomicrograph of a lower confocal section of the same specimen (merged image). Most cell bodies appear now sectioned longitudinally, as inferred from their elongated shape.

Since the vertical (anterior and posterior) canal cistae gave similar result, no differential analysis was performed.

RESULTS

Because of the complex morphology of the semicircular canal cistae, we created schematic representations to illustrate the plane of the slices. A representative image is shown in Figure 2A for a slice obtained by cutting the vertical ampullla parallel to the surface of the cista. The most central region consists of the eminentia cruciata (E.C.), which in the mouse is about 50 µm long (cista longitudinal axis) and 100 µm large (cista transverse axis), contains one or two layers of non-sensory cells and is devoid of hair cells and afferent and efferent innervation (Purcell and Perachio, 1997; Desai et al., 2005). The lateral areas of the cista are bounded by the planum semilunatum (P.S.), a non-sensory epithelium of semilunar shape with a high cellular density. Since the cista curves along the walls of the ampullla, confocal scanning from top to bottom will at first show the most lateral regions only, and then the rest of the cista. Moreover, since sensory and supporting cells in the cista are fan-like arranged, depending on the level of the confocal scanning they will appear sectioned mostly transversally (upper sections) or longitudinally (lower sections).

Figure 2B shows a representative photomicrograph of an upper section of the slice cut as shown in Figure 2A. Here and in the next images cell nuclei are labeled with DAPI (blue). Note that GAD67 (Figure 2C, green) is expressed in the peripheral zone (P.Z.) of the cista, whereas no staining is present in the central zone (C.Z.) nor in the E.C. Merge of Figures 2B,C is shown in Figure 2D. Most cells at this section level are cut transversally. Figure 2E shows a photomicrograph of a lower section of the same specimen. Note that, despite the impression that stained cells are now dispersed throughout the cista, because of the complex cista morphology as discussed above, they are actually located in the P.Z. only. To confirm this, we also performed transverse and longitudinal slices. Figure 3 shows two representative transverse sections from a same vertical cista. Consistent with the previous images, GAD67 expression was not detectable in the C.Z. (Figure 3C) and in the E.C. (Figure 3D). At this magnification the shape and location of the GAD67-positive cells is also clear and typical of supporting cells—see also Figure 4C below. Note in fact their small nuclear regions (red arrowheads), which are aligned and in contact with the basement membrane, and their thin, thread-like bodies running in-between the sensory cells prior to enlarge at the apical (luminal) surface. In contrast, the nuclei of sensory hair cells (yellow arrowheads) occupy the upper layer and are slightly staggered to form a pseudostratified epithelium.

Similar results were obtained by sectioning the vertical cistae longitudinally (Figure 4). Note that since the slice was through the...
slope of the crista (i.e., not medial), the supporting cells located at both sides of the E.C. (arrowhead in Figure 4B) were GAD67-positive, which is consistent with Figures 2, 3. A magnification of the same image is shown in Figure 4C.

Figure 5 shows the expression of GAD67 in a representative horizontal crista. Note that the morphology of the horizontal crista differs in not having the E.C. Like for the vertical cristae, however, GAD67 was found to be expressed by peripheral supporting cells only.

In some experiments, an antibody for calbindin was also used to show the calyx nerve terminals (Lysakowski et al., 2011). Figure 6 shows such an example in a horizontal crista; note that calbindin antibody (red) clearly stained several afferent calyces, mostly located in the central zone, where GAD67 was conversely absent.

A set of immunofluorescence labeling experiments was also performed to assess the expression of GAD65 in the crista sensory epithelium. However, we found no evidence for this isoform expression in either wild type (data not shown) or GAD67-GFP knock-in mice (Figure 7A). The functionality of the GAD65 antibody was previously assessed in the mouse cerebellum (Figure 7B).

Finally, in order to test for the possible co-localization of GAD67 and GABA in the sensory crista epithelium, we performed
a set of experiments in which GABA immunofluorescence was also tested in transgenic GAD67-GFP mice. As shown in Figure 8, GABA positivity was detected in all supporting cells, either expressing or not GAD67. No GABA expression was found in any hair cell.

By analyzing several confocal images of vertical and horizontal canal cristae, we were able to reconstruct the topographical distribution of GAD67- and GABA-expressing supporting cells, which is shown in Figure 9.

**DISCUSSION**

Adult animals express two isoforms of GAD, GAD67 and GAD65. Both GAD isoforms can synthesize GABA, but GAD67 might preferentially synthesize cytoplasmic GABA for metabolic purposes while GAD65 for vesicular release (Soghomonian and Martin, 1998). The present study provides the first evidence for the expression in mouse vestibular crista of GAD67, whereas GAD65 was not found. GAD67 expression was confined to peripherally-located supporting cells, which also expressed GABA. The co-expression of GABA and GAD67 suggests a role for this enzyme in converting glutamate released from hair cells into GABA for its metabolic oxidation.
exist in the crista. Recently, it has been reported that the same mRNA coding for GAD67 can generate 10 splicing isoforms, one of which produces an enzymatically active 44 kDa peptide (GAD44) (Trifonov et al., 2014). Also, excess GABA synthetized by peripheral supporting cells might be transferred to central supporting cells by plasma membrane GABA transporters (Ito et al., 2007; Roth and Draguhn, 2012) expressed by all supporting cells.

FUNCTIONAL MEANING OF GAD67 AND GABA EXPRESSION

The role of GABA in the vestibular epithelium has been matter of debate (Guth et al., 1998; Meza, 1998), and its possible involvement in vestibular signaling modulation remains to be ascertained. Our results indicate that neither GABA nor GAD are expressed by vestibular hair cells and thus it is possible to exclude a GABA involvement in afferent transmission. Here we found that all supporting cells, but not sensory cells, contain GABA. Moreover, GAD67, but not GAD65, was expressed in some supporting cells. In GABAergic neurons, GAD67 is found throughout the cell, whereas GAD65 preferentially localizes to synaptic terminals (Esclapez et al., 1994). Consistent with their subcellular localization, GAD67 provides both the cytoplasmic pool of GABA, which enters the tricarboxylic acid cycle to produce energy, and the vesicular pool of GABA (Tian et al., 1999; Lau and Murthy, 2012), while GAD65 primarily regulates the vesicular pool (Kaufman et al., 1991; Soghomonian and Martin, 1998), especially under conditions of sustained...
synaptic activity (Tian et al., 1999). Since vestibular supporting cells do not show a presynaptic machinery or vesicles, it seems reasonable to suggest that glutamate, once picked up by supporting cells, is converted into GABA for metabolic purposes. On the other hand, the possibility that GAD67 is also involved in some form of non-vesicular modulation (Soghomonian and Martin, 1998; Ito et al., 2007) of vestibular signaling cannot be excluded.

WHY DO ONLY PERIPHERAL SUPPORTING CELLS EXPRESS GAD67?

A major difference between the P.Z. and the C.Z. is the afferent innervation, as most complex calyces and calyx-only afferents are found in the C.Z. (Wersall, 1956; Lysakowski and Goldberg, 1997; Desai et al., 2005; see Figure 6 here), while bouton-only afferents are exclusively present in the P.Z. (Leonard and Kevetter, 2002). Bouton endings contact Type II hair cells, whereas calyx endings surround almost the entire basolateral surface of Type I hair cells. While in the case of Type II hair cells, clearance of glutamate involves its diffusion to supporting cells, in the case of Type I hair cells glutamate might be cleared from the calyx synaptic cleft by EAAT4 and EAAT5, two glutamate transporters recently found in Type I hair cells and calyx endings (Dalet et al., 2012). If glutamate released by Type I hair cells is cleared by EAAT4 and EAAT5, and glutamate released by Type II hair cells is cleared by GLAST expressed by the supporting cells, then why do only supporting cells located in the P.Z. express GAD67 given that Type II hair cells are also present in the C.Z.? One possible reason for the peripheral-only expression of GAD67 is that afferent nerve fibers innervating the P.Z. are characterized by a tonic discharge of action potentials, implying a continuous release of neurotransmitter, compared to the irregular firing of afferents from the C.Z. (Eatock and Songer, 2011). Glutamate, once picked up by supporting cells, can be converted into glutamine by glutamine-synthetase (Takumi et al., 1997). GAD67 could represent an additional mechanism for a more efficient metabolism of glutamate where its exocytosis is most abundant. It is important to consider that the rapid clearance of glutamate is required not only to restore the sensitivity of the system, but also to avoid glutamate cytotoxicity.

In conclusion, this is the first report of a marker that allows to distinguish peripheral supporting cells from central supporting cells in mammalian vestibular epithelia. This finding might be important for studies aimed at tracing cells lineage during vestibular epithelia regeneration, which is thought to occur by supporting cell transdifferentiation and/or mitosis (Rubel et al., 2013).

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Vertical crista

Horizontal crista

FIGURE 9 | Topographical distribution of GAD67 and GABA in the mouse vertical and horizontal cristae. Schematic representation showing the expression of GAD67 (green) by peripheral supporting cells as inferred by the confocal experiments. Red dots indicate supporting cells expressing GABA. C.Z.: central zone; P.Z.: peripheral zone; E.C.: planum semilunatum; E.C.: eminentia cruciata.

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