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Function coupling of otoferlin with GAD65 acts to modulate GABAergic activity

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Otoferlin, an integral membrane protein implicated in a late stage of exocytosis, has been reported to play a critical role in hearing although the underlying mechanisms remain elusive. However, its widespread tissue distribution infers a more ubiquitous role in synaptic vesicle trafficking. Glutamate, an excitatory neurotransmitter, is converted to its inhibitory counterpart, γ-aminobutyric acid (GABA), by L-glutamic acid decarboxylase (GAD), which exists in soluble (GAD67) and membrane-bound (GAD65) forms. For the first time, we have revealed a close association between otoferlin and GAD65 in both HEK293 and neuronal cells, including SH-SY5Y neuroblastoma and primary rat hippocampus cells, showing a direct interaction between GAD65 and otoferlin’s C₂ domain. In primary rat hippocampus cells, otoferlin and GAD65 co-localized in a punctate pattern within the cell body, as well as in the axon along the path of vesicular traffic. Significantly, GABA is virtually abolished in otoferlin-knockdown neuronal cells whereas otoferlin overexpression markedly increases endogenous GABA. GABA attenuation in otoferlin-knockdown primary cells is correlated with diminished L-type calcium current. This previously unknown and close correlation demonstrates that otoferlin, through GAD65 functional coupling, acts to modulate GABAergic activity. The discovery of otoferlin–GAD65 functional coupling provides a new avenue for understanding the molecular mechanism by which otoferlin functions in neurological pathways.

Keywords: otoferlin, GAD65, GABA, neurotransmitter, exocytosis

Introduction

Otoferlin is an integral membrane protein of ~1200 amino acids, which exists in both full-length and short isoforms that differentially utilize exons 47 and 48 (Yasunaga et al., 2000). Mutations in otoferlin have been implicated in hearing (Pangrsic et al., 2012); however, molecular mechanisms underlying otoferlin’s neuronal function remain elusive. In addition to a transmembrane (TM) domain, otoferlin contains multiple C₂ domains (3–6 termed C₂A–C₂F depending on the isoform), which are calcium (Ca²⁺)-binding motifs implicated in membrane trafficking (Yanez et al., 2012). The C₂F domain is reported to bind to syntaxin-1A and SNAP25 of the synaptic complex in a Ca²⁺-dependent manner; the C₂D domain binds to syntaxin-1A to a lesser extent, but also binds to the Ca₃.1.3 Ca²⁺-channel in a Ca²⁺-dependent manner (Ramakrishnan et al., 2009; Pangrsic et al., 2012). Mutations associated with deafness (P1825A in C₂F and L1011P in C₂D) were suggested to impair these interactions (Ramakrishnan et al., 2009). Other hypothetical roles of otoferlin have been reported in Ca²⁺-dependent vesicle priming, fusion, and replenishment (Pangrsic et al., 2012).

Early studies utilized sequence information from the short otoferlin isoform to demonstrate that otoferlin mRNA was restricted to inner hair cells (IHCs) and the vestibular system (Yasunaga et al., 1999, 2000; Judice et al., 2002), but a longer isoform could be found in the human adult brain (Yasunaga et al., 2000). Further studies revealed that, while the long isoform of otoferlin is restricted to the brain, short isoforms are widely distributed in fetus, adult brain, placenta, skeletal muscle, kidney, and heart (Varga et al., 2003). Moreover, within the brain, otoferlin is distributed in several regions including the cerebellum, hippocampus, and cortex. In contrast to central nervous system (CNS) synapses, IHC synaptic vesicles (SVs) are highly enriched with otoferlin, which is implicated in a late stage of exocytosis; otoferlin expression in IHCs correlates with afferent synaptogenesis and is localized to ribbon-associated SVs (Roux et al., 2006). Indeed, otoferlin has been shown to be a TM protein in IHC SVs, interacting with the SNARE complex proteins syntaxin-1 and SNAP25 (Roberts, 2006). Otoferlin is required for Ca²⁺-dependent exocytosis at vestibular hair cell ribbon synapses (Dulon et al., 2009), and has also been detected in mature outer hair cells responsible for low-frequency
processing and in auditory nerve fibres (Schug et al., 2006). Interestingly, its subcellular localization was found to be remote to sites of active release, seemingly contradicting its proposed role (Schug et al., 2006). As such, rather than being restricted to Ca\(^{2+}\)-triggered exocytosis, a more ubiquitous role in early/recycling endosome-T/S Andersen et al., 2009 has been proposed for otoferlin (Schug et al., 2006; Heidrych et al., 2008; Zak et al., 2011, 2012; Duncker et al., 2013).

While the presence of multiple C\(_2\) domains has led to investigations in otoferlin's role as a Ca\(^{2+}\)-sensor (Roux et al., 2006; Johnson and Chapman, 2010; Pangrsic et al., 2012), we focused on its potential correlations with neurotransmitters. It is known that otoferlin is located within vesicles and involved in exocytosis, implying a role in regulating neurotransmitter release. Moreover, mutations in OTOF, the human gene encoding otoferlin, result in a nonsyndromic form of deafness (DFN89) caused by abnormal neurotransmitter release from IHCs (Defourny et al., 2011). IHCs are known to release glutamate, with otoferlin potentially acting to regulate Ca\(^{2+}\)-triggered exocytosis of the neurotransmitter. This excitatory neurotransmitter, potentially related to tinnitus, causes GABA to decrease; deafness followed by tinnitus indicates a decrease in inhibitory neurotransmitter function. Furthermore, ~60%~75% of all synapses in the CNS are GABAAergic (Aljibri et al., 2008). These observations led us to investigate a potential connection between otoferlin and neurotransmitters.

The primary excitatory neurotransmitter glutamate (Defourny et al., 2011) is a key metabolite that is converted to an inhibitory neurotransmitter \(\gamma\)-aminobutyric acid (GABA) by \(\nu\)-glutamic acid decarboxylase (GABA) (Nedergaard et al., 2002), which exists in both membrane-associated (GAD65, 65 kDa) and soluble (GAD67, 67 kDa) forms in the mammalian brain. GAD65 is synthesized as a soluble protein (Reetz et al., 1991; Christgau et al., 1992) and lacks recognizable membrane-association sequences, although its N-terminal region has been implicated in membrane association (Fenali et al., 2007). Unlike GAD67, GAD65 undergoes palmitoylation, is activated by phosphorylation, and can be proteolytically cleaved, which is thought to decrease/abolish membrane association (Wei and Wu, 2008; Kanaani et al., 2010). GAD65 is transiently activated in response to demand for extra GABA in neurotransmission, being primarily responsible for GABA synthesis to serve as a neurotransmitter in vesicular mechanisms (Fenali et al., 2007). As a neurotransmitter, GABA plays an important role in the CNS with variations of its levels observed in different physiological conditions, including stroke (Clarkson et al., 2010). Both glutamate and GABA are stored in SVs and released through Ca\(^{2+}\)-dependent exocytosis (Nedergaard et al., 2002). Since GAD65 is membrane associated (Jin et al., 2003), and otoferlin is an integral membrane protein implicated in a late stage of exocytosis, we investigated a potential, functional relationship between the two (Dupuy and Houser, 1996; Schug et al., 2006). Herein, we demonstrate clear evidence revealing a physical and functional link between otoferlin and GAD65, which extends to attenuation of GABA and Ca\(^{2+}\) flux in otoferlin-knockdown cells. This novel discovery shows an unprecedented coupling between the two proteins by which otoferlin, through GAD65, modulates GABAergic activity.

**Results**

**Co-localization of otoferlin and GAD65**

Using double fluorescent immunolabelling in rat hippocampus tissue slices, we confirmed that both GAD65 and otoferlin are detectable in the hippocampus (Figure 1A) (Dupuy and Houser, 1996; Schug et al., 2006) and demonstrated prominent otoferlin and GAD65 co-localization in the CA1 and CA2 regions and other sections, including the cortex and grey matter (Figure 1B–E). Enzyme immunoassay (EIA) experiments on rat brain lysates demonstrated the presence of GAD65 in the formed immunocomplex using an antibody against otoferlin (Figure 1F), suggesting otoferlin and GAD65 may interact with each other. Further, western blot analyses on SVs isolated from rat brain revealed the presence of both proteins in this subcellular fraction (Figure 1G).

Subsequently, we performed similar immunocytochemistry experiments on primary rat hippocampus cells and demonstrated co-localization of endogenous otoferlin and GAD65 in the cell body, as well as, punctate co-localization along the axon (Figure 2), which corroborates with GABA vesicular traffic. RT–PCR analyses confirmed significant expression of both GAD65 and otoferlin in these cells (Supplementary Figure S1).

To investigate this potential interaction further, confocal studies were performed on HEK293 cells transiently transfected with constructs encoding CFP-otoferlin and YFP-GAD65 (Supplementary Figure S2), respectively. The result revealed clear co-localization of the two proteins at the plasmalemma, with the otoferlin-associated signal showing a punctate pattern (Figure 3A). Co-iP experiments on cell lysates from HEK293 cells transfected with CFP-otoferlin derivatives and/or YFP-GAD65 also supported the interaction of both full-length and truncated otoferlin derivatives with GAD65 (Figure 3B). We then focused on the C\(_2\)D, C\(_2\)E, and C\(_2\)F domains, as mutations therein have been closely connected with hearing loss. Cells transfected with the C-terminal fragment of otoferlin, comprising the C\(_2\)E and C\(_2\)F domains fused with the TM domain, showed a similar phenocopy in both confocal microscopy and fluorescence resonance energy transfer (FRET) (Figure 3C). Further analyses using a derivative only containing the C\(_2\)F domain with the TM also indicated co-localization with GAD65 (Supplementary Figure S3).

We further used FRET to examine the relationship between otoferlin and GAD65. Utilizing CFP-otoferlin as the fluorescence donor and YFP-GAD65 as the acceptor, FRET signal was revealed at the plasmalemma of the cells with a FRET efficiency close to ~80% (Figure 4A and B). The FRET signal was drastically decreased and membrane-associated signal virtually abolished (Figure 4A and B) when cells were transfected with a truncated otoferlin (TO) construct lacking the TM domain (Supplementary Figure S2). It is widely known that GAD65 is expressed as a cytosolic soluble protein that is ultimately translocated to the membrane (Reetz et al., 1991; Christgau et al., 1992). The membrane anchoring of GAD65 remains elusive with previous reports of its membrane interaction involving ionic (Fornum, 1968), hydrophobic interactions (Chang and Gottlieb, 1988), protein phosphorylation (Namchuk et al., 1997), or even the formation of a complex with SV-related proteins (Jin et al., 2003). Our results demonstrate that otoferlin may facilitate this membrane association. Interestingly, a truncated
GAD65 (TG) construct lacking the first 83 N-terminal amino acids implicated in membrane association (Fenalti et al., 2007) decreased the FRET efficiency from \( \approx 80\% \) to \( \approx 50\% \), yet still exhibited, to some extent, membrane co-localization with co-transfected full-length otoferlin isoform that includes the C2D, C2E, and C2F domains (Figure 4A and B). Thus, it is likely that there are other elements outside the N-terminal region of GAD65 involved in targeting it to the membrane, possibly mediated by otoferlin C2 domain(s). This is corroborated by the observation of FRET signal when both truncated derivatives of otoferlin and GAD65 are present (Figure 4A and B). Furthermore, the decrease in signal in the presence of the truncated GAD65 implies a potential role of the N-terminal domain of GAD65 in mediating binding interactions with otoferlin.

**Otoferlin and GAD65 interact in vitro**

We next investigated whether recombinant, purified otoferlin and GAD65 can interact directly in vitro. Purification of the otoferlin C2 domains expressed in *E. coli* tends to be difficult due to low solubility (Pangrsic et al., 2012). However, we successfully expressed and purified two individual C2 domains (C2E and C2F, respectively) by fusing each to a SUMO tag and utilizing low-temperature expression (10°C–15°C) in bacterial cells. In addition, we found that co-expression and/or co-purification of otoferlin derivatives in the presence of GAD65 facilitates the purification of both proteins. Using GST-pulldown assays, we observed that both C2 domains interacted with GAD65 (Figure 4C–E), consistent with the confocal results (Figure 3 and Supplementary Figure S3). Although these C2 domains are implicated in Ca\(^{2+}\) binding, it should be noted that, to
date, we have not found any effect of Ca$^{2+}$ on complex formation by size exclusion chromatography (data not shown).

**Effect of otoferlin on GAD65 activity**

Once the interaction between otoferlin and GAD65 was established, we investigated whether this physical association translates to the functional level. HEK293 cells were used to create stable cell lines in which endogenous otoferlin expression was knocked down to $\sim$20% by transfection with otoferlin shRNA (Figure 5A, left), or otoferlin was overexpressed using the pECFP-otoferlin vector (Figure 5A, right). Western blot analyses were performed on synaptosomal preparations that enabled the detection of the low levels of endogenous otoferlin in wild-type cells (Figure 5A, left). Overexpression of otoferlin in these cells resulted in a concomitant increase in GAD65 expression (Figure 5A, right). These observations were corroborated at the RNA level by semi-quantitative RT–PCR, which demonstrated that changes in otoferlin overexpression caused concomitant changes in GAD65 expression without affecting GAD67 (Figure 5B). Similarly, GAD65 overexpression was correlated with an increase in otoferlin expression (Figure 5B, right panel). Given the low endogenous level of otoferlin in these cells, a nested PCR strategy is needed to amplify detectable amounts of otoferlin.

Given the role of GAD65 in GABA production, as well as the co-localization of otoferlin with GAD65 along the path of vesicular traffic, we sought to explore whether otoferlin was involved in the regulation of this neurotransmitter. We performed an ELISA assay to investigate the effects of modulated otoferlin expression on GABA levels in HEK293 cells. As the positive control, GAD65 overexpression resulted in a significant increase in GABA levels in cells. Interestingly, otoferlin overexpression also increased GABA substantially, demonstrating its direct effect on GAD65 function. In contrast, little or no GABA was observed in stable otoferlin-knockdown cells (Figure 5C).

Although we have confirmed that all main SNARE genes are expressed in HEK293 cells (Supplementary Figure S4), we sought to test the effect of otoferlin–GAD65 coupling further in a more relevant neuronal environment. SH-SY5Y neuronal cells
were transfected with otoferlin shRNA to create a stable, otoferlin-knockdown cell line for functional studies; control SH-SY5Y cells were stably transfected with control A shRNA. Considering the very low endogenous expression of GAD65 in SH-SY5Y cells, the nested PCR strategy was utilized to demonstrate the attenuation of GAD65 expression when otoferlin expression was knocked down, while not changing GAD67 expression (Figure 6A). Subsequently, GABA levels in culture medium from

**Figure 3** Confocal and co-IP studies using full-length and truncated derivatives of otoferlin show that otoferlin interacts with GAD65 via its C2 domains. (A) Confocal microscopic images of HEK293 cells transiently transfected with full length CFP-otoferlin isoform 4 (C2-D-E-F as indicated in Supplementary Figure S2, cyan) and YCP-GAD65 (yellow), showing co-localization of the two (Overlay). Merged images represent the merging of bright field and overlayed images. (B) Co-IP of otoferlin and GAD65 in lysates from HEK293 cells transfected with otoferlin isoform 4 (CFP-C2-D-E-F) and/or YFP-GAD65 by using the indicated antibodies. Western blot analyses were performed on the immunocomplexed proteins using an antibody against GFP that also recognizes the CFP and YFP mutants to probe. Samples in lanes 1, 4, and 7 are cell lysates from the respective HEK293 cells as positive controls. Samples in lanes 3 and 10 are cell lysates immunoprecipitated with normal IgG as negative controls. (C) Confocal microscopic images of HEK293 cells transiently transfected with a truncated CFP-otoferlin derivative containing two C2 domains (C2-E-F) fused to the TM domain (cyan) and YCP-GAD65 (yellow), showing co-localization as in A. FRET analyses were also performed, with FRET efficiencies indicated in the adjacent colour bar, further supporting the co-localization.
knockdown and control SH-SY5Y cells were quantified daily over a period of 4 days by ELISA. In comparison with the control cells, otoferlin knockdown in SH-SY5Y cells resulted in a significant attenuation of GABA production, with little or no GABA detected by ELISA (Figure 6B and C). Thus, changes in GABA production are specifically linked to changes in GAD65—but not GAD67-expression.

Subsequently, we performed similar studies on primary rat hippocampus cells, to exclude any incorporation of artificial characteristics in clonal cell lines due to repetitive passaging (Coles-Takabe et al., 2008). E18 Sprague Dawley rat hippocampus dissociated cells were utilized, providing a context closer to the in vivo situation. GABA production was quantified in non-transfected cells, cells transfected with control siRNA, and cells in which otoferlin expression was knocked down by otoferlin siRNA. Similarly, knockdown of otoferlin in the primary hippocampus cells resulted in a significant attenuation (>50%) of GABA production (Figure 7A). This result is extremely intriguing as it clearly demonstrates that the established close relationship between otoferlin and GAD65 translates to the functional level by affecting GABA levels in primary neuronal cells.

We next examined whether otoferlin could affect GABA-involved electrophysiological activity in primary hippocampus cells. To this end, we employed whole-cell patch clamp to measure depolarization-induced Ca$^{2+}$ currents ($I_{Ca}$) in hippocampus using Ba$^{2+}$ as the charge carrier (Figure 7B–D). A typical example of Ba-carried $I_{Ca}$ was recorded in primary rat hippocampus cells at 24 h after transfection with control siRNA or otoferlin siRNA (Figure 7B). Further analysis of the peak inward current at each voltage and the summarized peak inward current recorded at -10 mV demonstrated an ~37% attenuation of Ca$^{2+}$ flux ($I_{Ca}$) in cells transfected with otoferlin siRNA (Figure 7C and D). These results demonstrate the direct impact of otoferlin on Ca$^{2+}$ influx. Given that Ca$^{2+}$ current is associated with GABA receptor activation, we speculate that the attenuation of GABA levels as a result of otoferlin knockdown with concomitant reduction of GAD65, may lead to a diminished activation of GABA receptors and subsequent reduction in Ca$^{2+}$ influx. Preliminary experiments using nifedipine, a dihydropyridine Ca$^{2+}$-channel blocker that primarily blocks L-type Ca$^{2+}$-channels, showed that treatment with higher concentration of nifedipine resulted

Figure 4 FRET and pulldown analyses of otoferlin and GAD65 interaction. (A) FRET analyses of HEK293 cells transiently transfected with constructs for CFP-otoferlin and YFP-GAD65 (O-G), CFP-otoferlin and YFP-truncated GAD65 (O-TG), CFP-truncated otoferlin and YFP-GAD65 (O-G), or truncated derivatives of both (TO-TG), as described in Supplementary Figure S2. (B) FRET efficiency analyses of the aforementioned experiments (n = 5). (C–E) GST-pulldown assays using SUMO-GAD65 and GST (C), GAD65 and the otoferlin C2E domain (D), and GAD65 and the otoferlin C2F domain (E). For C, lane 1: whole cell lysis; lane 2: supernatant; lane 3: flow-through; lane 4: elution. For D and E, lane 1: elution of GAD65 and otoferlin C2E or C2F from Ni-NTA Agarose; lane 2: following overnight dialysis and SUMO protease digestion, the His-SUMO tag was removed by Ni-NTA Agarose; lane 3: elution with 5 mM reduced glutathione; lane 4: elution with 10 mM reduced glutathione.
in lower GABA levels (Figure 7E), which was similar to previous observations in brainstem regions of the rat (Kubo et al., 1990), suggesting a potential link between GABA and L-type Ca\(^{2+}\)-channels. However, much work is needed to fully elucidate any potential relationship. Taken together, this newly discovered otoferlin–GAD65 coupling directly connects changes in otoferlin expression to GABA production and may extend to L-type Ca\(^{2+}\) flux.

**Figure 5** Expression of otoferlin and GAD65 in HEK293 cells is correlated. HEK293 cells were stably transfected with recombinant vectors pECFP-otoferlin for otoferlin overexpression, and pEYFP-GAD65 for GAD65 overexpression. Control cell lines were transfected with pECFP-C1, and pEYFP-N1, respectively. (A) Western blot analyses were performed on synaptosomal preparations from otoferlin control and knockdown cells to detect otoferlin (left) and cell lysates from control and otoferlin-overexpressing cells to detect otoferlin and GAD65 (right). β-actin was probed as an internal control. Otoferlin: 140 kDa; Otoferlin + CFP: 167 kDa; GAD65: 65 kDa. Densitometry results for gels are shown below each respective gel. Quantity One 1-D Analysis Software (Version 4.4) (Bio-Rad Laboratories) was used to quantify the intensity of each protein band. The intensities of protein bands of interest were normalized to β-actin to generate a relative expression ratio. (B) Semi-quantitative RT–PCR analyses were performed on RNA extracted from the indicated HEK293 cell lines to examine otoferlin (217 bp), GAD65 (467 bp), and GAD67 (626 bp) expression levels. β-actin was amplified as an internal control. A nested PCR strategy was utilized to detect low levels of endogenous otoferlin. (C) GABA levels in various HEK293 cell lines, as well as their respective controls, were quantified by ELISA. The amount of GABA was quantified using a standard curve of purified GABA. Bars represent mean ± STD. *P < 0.001 relative to its respective control.
Discussion

In this study, we demonstrate that otoferlin and GAD65 are specifically and functionally coupled, in an intimate relationship involving correlation of expression at both RNA and protein levels. This coupling is the conduit by which otoferlin can act to modulate GABA production within the SVs that, in turn, affects Ca\(^{2+}\) flux at synapses. GABA regulates neuronal activity by modulation of ion channels through the activation of GABA receptors (Catterall, 2000; Dolphin, 2003; Tedford and Zamponi, 2006). It is well known that GABA receptor activation can lead to the enhancement of L-type Ca\(^{2+}\) current in hippocampal cultures obtained from neonatal rat pups (Carter and Mynlieff, 2004; Bray and Mynlieff, 2009; Bray and Mynlieff, 2011).

Our initial studies were performed in the HEK293 cell line that, although designated as a kidney cell line, has a neuronal lineage...
expressing several proteins typically found in neurons (Shaw et al., 2002) while exhibiting the Ral and phospholipase D2-dependent pathway for constitutive metabotropic glutamate receptor endocytosis (Bhattacharya et al., 2004). Previous studies in HEK293 cells have revealed the expression of mRNAs associated with SVs and electrically excitable membranes (Shaw et al., 2002). This, and the hardness of this cell line, made it a good context for our investigation to provide proof-of-principle. Having established the intimate coupling between otoferlin and GAD65 in these cells through multiple approaches, the next logical step was to test the relationship further in a more relevant neuronal environment. SH-SY5Y is a clonal neuroblastoma cell line that can make dopamine, acetylcholine, as well as GABA under certain conditions or passages (Biedler et al., 1978). But they are not as robust as the HEK293 cells, thus requiring very careful handling and maintenance. Finally, the ability to replicate these observations in the more delicate primary rat hippocampus cells, allowed us to definitively confirm our observations without the possibility that these are artificial characteristics incorporated due to repetitive passaging as is sometimes observed with clonal cells.

The simultaneous reduction of GABA production and Ba~currents in hippocampal cells transfected with otoferlin shRNA does not, in itself, directly prove that otoferlin and GAD65 functionally interact with each other. However, they are pieces of a puzzle we intend to solve. Further experiments using specific molecules to block the relationships involved in the observed phenomena.

Otoferlin is essential for a late step of exocytosis including vesicle priming and fusion (Pangrsic et al., 2012). Our results reveal a close correlation between otoferlin levels and GABA production, which was completely unknown previously. This novel finding demonstrates that GAD65 is intimately coupled to otoferlin’s function in exocytosis to modulate GABAergic activity. More than 40 amino-acid mutations in otoferlin, including those in the TM, have been implicated in human deafness (Pangrsic et al., 2012). Furthermore, there is a potential connection between a decrease in GABAergic neurotransmission and clinical pathogenesis in several neurological disorders including some forms of epilepsy, chronic pain, anxiety, and other mood disorders (Asada et al., 1996; Wong et al., 2003). Indeed, ~60%–75% of all synapses in the CNS are GABAergic (Aljabri et al., 2008). Moreover, it has been observed that, in the auditory system, expression of the vesicular glutamate transporter 3 (VGLUT3) and glutamate co-transmission are prominent in the development of the GABA/glycinergic sound-localization pathway with glutamate co-transmission being crucial for synaptic reorganization and topographic specification of a developing inhibitory circuit (Noh et al., 2010). Interestingly, there have been a few populations of neurons observed to release both glutamate and GABA, including granule cells in the dentate gyrus and neurons in the auditory brainstem (Seal and Edwards, 2006). Since GAD is involved in conversion of glutamate to GABA (Nedergaard et al., 2002), it is possible that otoferlin may be involved in regulating the balance between these two contrasting neurotransmitters through its relationship with GAD65. Thus, our discovery of the unprecedented otoferlin−GAD65 functional coupling provides a new avenue for understanding the molecular mechanism by which otoferlin functions in hearing (Parsons, 2006) and other neurological pathways.

Given that GABA levels correlate with otoferlin expression, otoferlin−GAD65 coupling appears to suggest a new means to modulate GABAergic activity in neuronal cells. Significantly, this novel observation provides a new direction for further understanding otoferlin’s role in neuronal function, which largely remains unclear.

Materials and methods

Immunohistochemistry

All animal care and treatment were performed in accordance with the Canadian Council of Animal Care Regulations and approved by Queen’s University Animal Care Committee. Rats were anaesthetized with sodium pentobarbital (70 mg/kg, intraperitoneal) and transorally perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed, post-fixed in the same fixative solution for 1 h at 4 °C, and then cryoprotected in 30% sucrose in PB for 48 h at 4 °C. Brain sections (40 μm) were cut on a freezing sledge microtome and collected in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Sections were washed 3 × 5 min in 0.1 M PBS followed by incubation with 5% normal goat serum (NGS) in PBS-T (PBS and 0.2% Triton X-100) for 2 h at room temperature. Sections were then incubated for 48 h at 4 °C with anti-GAD 65 (1:500, SYSY 198102) and anti-otoferlin (1:200, Abcam ab53233). After 3 × 10 min washes in PBS-T, sections were incubated in the dark for 2 h at room temperature with the appropriate secondary antibodies conjugated to Alexa fluorophores (488 or 594 nm, Molecular Probes, Invitrogen) at a 1:200 dilution in 1% NGS in PBS. Finally, sections were washed 3 × 10 min in PBS prior to mounting onto glass slides, then cover-slipped with Aquamount. Brain sections were examined, and images were obtained using confocal microscopy (Leica).

Co-immunoprecipitation and isolation of SVs

To prepare whole brain lysates for co-IP experiments, one rat was decapitated and the whole brain was removed. The brain was washed thoroughly with homogenization buffer including 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA (pH 8.0), 0.5% Triton X-100, 1 mM PMSF, and 1× protease inhibitor (Roche, 05056489001), and homogenized using a tight-fitting glass-Teflon homogenizer. The homogenate was centrifuged at 10000 × g for 10 min at 4 °C and the supernatant was collected. For co-IP studies, the appropriate antibody was added followed by Protein A/G PLUS-Agarose for immobilization according to the company’s protocol (Santa Cruz: sc-2003). For co-IP of the complex from transfected cells, RIPA buffer was used. Western blot analyses were performed using the GFP antibody (ab291) as a probe. Isolation of SVs from rat brain was performed as previously
described (Ahmed et al., 2013).

**Culture of rat hippocampal neuronal primary cells**

Embryonic day 18 (E18) Sprague Dawley rat hippocampus dissociated cells were obtained from BrainBits® and handled according to manufacturer’s directions. Cells were dispersed, counted, and diluted to an appropriate concentration with NbActiv1 (BrainBits). Cells were seeded onto 24-well plates coated with poly-ω-lysine (15 ml/cm²), with 32000 cells (16000 cell/cm²) seeded per well. Subsequently, plates were placed in a humidified incubator at 37°C containing 5% CO₂. Cells were maintained by replacing half of the medium with fresh, 37°C, 5% CO₂, equilibrated NbActiv1 every 3–4 days.

**Immunocytochemistry of rat hippocampal neuronal primary cells**

E18 hippocampus neurons were cultured in 24-well plates on coverslips coated with 50 μg/ml poly-ω-lysine (Sigma P6407). After 7 days, cells were washed with PBS to remove the culture medium, fixed with 4% paraformaldehyde at room temperature, and subsequently washed with PBS. Fixed cells were blocked by incubating in PBS containing 5% BSA, 5% NGS, and 0.1% Triton X-100 at room temperature for 2 h. Cells were then incubated overnight at 4°C with antibodies against otoferlin (1:200) and GAD65 (1:2500) in PBS containing 1% NGS and 1% BSA. Cells were washed with PBS and incubated with goat anti-rabbit and goat anti-mouse secondary antibodies conjugated to Alexa 594 and Alexa 488 fluorophores (1:200), respectively, for 2 h at room temperature. Finally, cells were washed with PBS and mounted onto slides and stored in the dark at 4°C prior to imaging.

**Constructs and FRET**

Constructs for GAD65 (BC126327), otoferlin isoform 4 (NM_194332) and its truncation (TO) lacking the TM domain have been generated using pEYFP-N1 and pECFP-C1, respectively. Primers utilized to construct recombinant GAD65 and otoferlin plasmids are described in Supplementary material.

For FRET, all plasmids were prepared using QiAfilter Plasmid Maxi kit after sequence confirmation. The HEK293 cell line was maintained in DMEM medium (Sigma), containing 10% (v/v) heat-inactivated fetal calf serum (Sigma). After transfection (Lipofectamine 2000 Transfection Reagent, Invitrogen), all cells were cultured in Delta T dishes (0.17 mm, clear, Biopotech) for 36 h under humidified air containing 5% CO₂. FRET images of live cells were captured through confocal microscopy and subjected to FRET efficiency analyses. FRET was acquired under Acceptor Bleaching mode (Leica LCS software) and using polygon region of interest (ROI) mode. The acceptor bleached FRET efficiencies are calculated using the following equation:

\[
\text{FRET}_{\text{eff}} = \frac{D_{\text{post}} - D_{\text{pre}}}{D_{\text{post}}} \quad \text{for all } D_{\text{post}} > D_{\text{pre}}
\]

**GST-pulldown**

Otoferlin (C2,E or C,F fused with a C-terminal GST-tag) and GAD65 were expressed using the pETSUMO system (Invitrogen) and co-purified using Ni-NTA Agarose (QIAGEN). After overnight dialysis and SUMO protease digestion, the His-SUMO tag was separated from the cleaved protein over Ni-NTA Agarose and the flow-through was utilized for subsequent GST-pulldown assays using Glutathione Superflow Agarose (Thermo Scientific Pierce). Furthermore, co-purified proteins (containing the SUMO tag), load (co-purified proteins after proteolytic cleavage of the SUMO tag), flow-through, wash, and eluate fractions were resolved by SDS–PAGE.

**Gene interference**

A recombinant plasmid containing otoferlin shRNA (Santa Cruz Biotechnology) was transfected into HEK293 or SH-SY5Y cells to knock down otoferlin gene expression using either Lipofectamine 2000 or 3000 Transfection Reagent (Invitrogen), and then selected by puromycin for 5 days. In parallel control experiments, cells were transfected with control A plasmid (Santa Cruz Biotechnology). The human otoferlin shRNA plasmid (sc-61269-SH) is provided as a pool of three different shRNA plasmids designed to maximize knockdown efficiency, including sc-61269-SHA, sc-61269-SHB, and sc-61269-SHC. Details are described in Supplementary material.

To create otoferlin-knockdown primary hippocampus cells, transient transfections were performed on the primary rat hippocampus dissociated cells (BrainBits) after 6 days following cell seeding. Prior to transfection, 0.2 ml of the media was removed and replaced with fresh NbActive1, and the cells were incubated for 16 h. For transfection, 20 pmol of otoferlin siRNA (Santa Cruz) was diluted into BrainBits transfection medium, and 1 μl Lipofectamine 2000 was also diluted into the medium, each with a final volume of 50 μl. The two diluted solutions were then combined to form a DNA/Lipofectamine complex that was subsequently equilibrated in an incubator (37°C, 5% CO₂) for 10 min. Transfections were performed for 4 h or overnight. After replacement of 90% of the medium with 37°C, 5% CO₂-equilibrated NbActive1, the culture plates were returned to the incubator for 48–72 h prior to analysis.

**Semi-quantitative RT–PCR**

For gene expression analyses, total RNA was isolated using the RNeasy Microarray tissue mini kit (Qiagen), and reversely transcribed to cDNA by OligoT-priming using SuperScript II reverse transcriptase (Invitrogen). For semi-quantitative PCR, the high fidelity PCR system (Roche) was utilized to amplify the fragment of interest from the cDNA template (1 μg) using the appropriate primers (0.3 μM) in a 50 μl reaction volume. A nested PCR strategy was required for otoferlin with the primers nFP and nRP, and for GAD65 amplification from SH-SY5Y cells using the primers n1FP and n1RP. Primers are described in Supplementary material.
Western blot analyses

All cell samples, including wild-type and vector-transfected HEK293 and SH-SYSY cells, were washed twice with PBS after overnight culture in humidified air containing 5% CO₂, and then lysed in RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology) using 1 ml RIPA per 1.0 × 10⁵ cells. Whole cell lysates were harvested by centrifugation at 4°C for 10 min at 12000 × g, and then denatured at 95°C for 5 min in 1 x SDS sample buffer before resolution on SDS–PAGE for western blot analyses.

For comparison of otoferlin protein in otoferlin-knockdown cells with control cells, western blot analyses were performed on sympathetic proteins extracted using Thermo Scientific Syn-Per Reagent using a modified protocol optimized for enrichment of SVs from cultured cells. In brief, media were decanted from cultured cells and the cells were washed twice with ice-cold PBS prior to the addition of 1 ml Syn-Per Reagent including 1 x complete EDTA-free protein inhibitor cocktail (Thermo Scientific, Roche) per 100 mm plate. Cells were scraped and the lysate from 3 x 100 mm plates was transferred to a microcentrifuge tube. Lysates were centrifuged at 12000 × g for 10 min at 4°C and the resultant supernatant was transferred to a new tube and further centrifuged at 15000 × g for 20 min at 4°C. The synaptosome pellet was resuspended in 2 x SDS loading buffer for western blot analyses.

Protein samples were resolved by SDS–PAGE. After being transferred to nitrocellulose membranes (GE Healthcare), proteins of interest were probed with the appropriate, respective antibodies at concentrations recommended by the manufacturers, including β-actin (sc-81178) and goat anti-mouse IgG-HRP (sc-2005), otoferlin (F-18: sc-50160) and donkey anti-goat IgG-HRP (sc-2020), GAD65 (A01496, GenScript) and goat anti-rabbit IgG-HRP (sc-2004). Blots were developed using a Luminata crescendo western HRP substrate kit (Millipore). The protein bands were visualized after exposure of the membranes to X-ray film (Kodak).

GABA quantification by ELISA

After shRNA transfection, HEK293 cells were cultured in T25 flasks for 36 h in humidified air containing 5% CO₂, followed by selection using G418 (Sigma) or puromycin for 6–7 days. Cells were seeded at the density of 1 x 10⁵ cells/ml in 96-well plates and cultured for 24 h. Cell culture media were harvested at 1000 rpm for 5 min and GABA was measured by ELISA (GABA Research ELISA, BA E-2600, Labor Diagnostika Nord) according to manufacturer’s protocol with reactions monitored. Quantification was performed by measuring the absorbance at 450 nm following the reaction and comparing with a standard curve prepared with known amounts of GABA.

After transfection with otoferlin shRNA or control vector and subsequent selection by puromycin, 100 µl of SH-SYSY cells at the density of 5 x 10⁵ cells/ml (i.e. 50000 cells) were plated into 96-well plates. Cells were incubated at 37°C in the presence of 5% CO₂, and a time course analysis was performed in triplicate to quantify GABA daily over a span of 4 days.

Primary rat hippocampus cells were transiently transfected with either otoferlin siRNA or control siRNA. Quantification of GABA was similarly performed according to manufacturer’s protocols (Labor Diagnostika Nord), using a standard curve prepared with a larger range of GABA (25–2500 ng/ml).

Electrophysiological recordings

Whole-cell voltage-activated Ca²⁺ currents were recorded in wild-type and otoferlin-knockdown primary rat hippocampus cells (BrainBits) using Ba²⁺ as the charge carrier. The pipette solution contained 135 mM CsCl, 5 mM MgATP, 10 mM EGTA, and 10 mM HEPES, and the pH was adjusted to 7.2 with CsOH. The bath solution contained 140 mM TEACl, 5.4 mM BaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, and the pH was adjusted to 7.4 with CsOH. The current was recorded using a 400 ms step protocol to voltages between −50 mV and 40 mV in 10 mV increments from a holding potential of −60 mV. The amplitude of the peak inward current at −10 mV was used to measure the inward current amplitude of native i_cl. Patch clamp experiments were performed at room temperature (22°C ± 1°C).

Statistics

Data were expressed as mean ± STD. A two-tailed paired Student’s t-test was used to determine the statistical significance between the control and experimental groups; a P-value of 0.05 was considered significant.

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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