Journal Club

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Deciphering the Roles of C₂-Domain-Containing Proteins (Synaptotagmins and Otoferlin) in the Inner Ear

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Review of Beurget et al.

Synaptic vesicle exocytosis is a highly regulated Ca²⁺-dependent form of SNARE-mediated membrane trafficking. The short time interval between presynaptic Ca²⁺ entry and vesicle fusion is made possible by high-affinity Ca²⁺-sensitive proteins that trigger exocytosis. Synaptotagmins (Syts) are a family of transmembrane SNARE accessory proteins that contain two cytoplasmic C₂-domains, which bind Ca²⁺, phospholipids, and SNARE proteins. Based on biochemical and functional findings, Syt1 and Syt2 (and recently Syt9) have become regarded as the primary Ca²⁺ sensors for exocytosis at neuronal synapses (Pang and Südhof, 2010). Surprisingly, however, neither Syt1 nor Syt2 was identified in hair cells of the inner ear (Safieddine and Wenthold, 1999). This suggested that a different (non-Syt) Ca²⁺ sensor triggers exocytosis of the specialized sensory synapses of hair cells. Because Syts are evolutionarily conserved in sequence and func-

tion throughout vertebrate and invertebrate species (Pang and Südhof, 2010), discovering a non-Syt-mediated mechanism for triggering Ca²⁺-dependent exocytosis at a neuronal synapse would be highly significant.

Genetic screening for a hereditary form of prelingual, nonsyndromic deafness (DFNB9) identified a mutation in otoferlin, a C₂-domain-containing protein (Yasunaga et al., 1999). Several findings led to the proposal that otoferlin is the hair cell-specific Ca²⁺ sensor for exocytosis. Otoferlin binds to SNAREs and Ca²⁺ channels, and five of otoferlin's six C₂-domains stimulate membrane fusion in a Ca²⁺-dependent manner with affinities similar to Syts (Roux et al., 2006; Ramakrishnan et al., 2009; Johnson and Chapman, 2010). Furthermore, genetic ablation of otoferlin results in severe hearing impairment resulting from improper hair cell synaptic development and lack of synaptic vesicle exocytosis (Roux et al., 2006; Heidrych et al., 2009). In addition, immature outer hair cell (OHC) exocytosis is otoferlin dependent and shows linear Ca²⁺ dependence (Beurget et al., 2008). Finally, in vestibular hair cells, otoferlin is required to achieve the linear dependence of exocytosis on Ca²⁺, supporting its role as a Ca²⁺ sensor (Dulon et al., 2009). Combined with the dearth of Syt1 and Syt2, these results suggest otoferlin triggers exocytosis of small, clear core vesicles in hair cells.

The role of otoferlin as the Ca²⁺ sensor for exocytosis in hair cells, however, has

been challenged. First, yeast-two-hybrid screens identified Rab8b GTPase and myosin VI as otoferlin-interacting partners (Heidrych et al., 2008, 2009), implicating otoferlin in endocytosis and transport of vesicles to active zones. Moreover, a role of otoferlin in vesicle priming for exocytosis has been proposed based on demonstration of insufficient vesicle replenishment in inner hair cells (IHCs) of mice carrying a point mutation in the C₂F domain (Pangrsic et al., 2010). Therefore, otoferlin may be involved in vesicle recycling. Because exocytosis, endocytosis, and vesicle recycling are highly interdependent components of the synaptic vesicle cycle, it is difficult to distinguish in which components otoferlin participates. Second, the broad distribution of otoferlin throughout hair cells challenges the notion of preferential sorting to synaptic vesicles and leads to speculation of a more general cell biological relevance (Schug et al., 2006; Goodyear et al., 2010). However, it is important to note that a similar distribution is observed for other synaptic vesicle proteins such as Vglut3 (Ruel et al., 2008; Seal et al., 2008; Pangrsic et al., 2010) and cysteine string protein (Schmitz et al., 2006). Third, Syt1 and Syt2 have recently been identified in IHCs (Beurget et al., 2010; Johnson et al., 2010), potentially rendering moot the quest for an alternative Ca²⁺ sensor. Fourth, otoferlin was not detected in IHCs in a hypothyroid rat model, but these cells ex-

Received Dec. 7, 2010; revised Dec. 25, 2010; accepted Dec. 29, 2010.

F.D.G. is a fellow in the FIRST teaching program at Emory University. P.M.Q. is supported by a National Institutes of Health minority supplement (National Institute on Deafness and Other Communication Disorders: DC007678). We thank Dr. Marlies Knipper, Christoph Franz, and Dr. Tobias Moser for personal communications. We also thank Dr. Felix E. Schweizer, Dr. Tobias Moser, and D. H. Naomi Quiñones for comments on the manuscript.

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DOI:10.1523/JNEUROSCI.6359-10.2011

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hibited Ca^{2+} -dependent exocytosis (Brandt et al., 2007; Johnson et al., 2010). Fifth, mouse models with otoferlin deficits show little vestibular dysfunction (Roux et al., 2006, 2009; Longo-Guess et al., 2007; Schwander et al., 2007; Dulon et al., 2009) even though otoferlin is normally expressed in auditory and vestibular hair cells (Schug et al., 2006; Goodyear et al., 2010). Sixth, the Ca^{2+} dependence of exocytosis changes from nonlinear to linear along the cochlea without qualitative changes in otoferlin expression (Johnson et al., 2009). Together, these observations implicate otoferlin in additional functions in the vesicle cycle, although they do not exclude the potential for otoferlin to trigger exocytosis.

Because of this ongoing controversy, Beurg et al. revisit the role of the Ca^{2+} -binding proteins (Syts and otoferlin) in facilitating Ca^{2+} -dependent synaptic vesicle exocytosis from cochlear hair cells in a recent article in *The Journal of Neuroscience* (Beurg et al., 2010). Beurg et al. (2010) elucidate an otoferlin- and Syt-independent phase of vesicle release that occurs in the first few postnatal days, raising the possibility of yet another Ca^{2+} sensor at hair cell synapses. Moreover, the authors argue that their evidence supports otoferlin's role as the Ca^{2+} sensor for exocytosis after this period. However, they do not exclude the possibility of an alternative role of otoferlin.

Beurg et al. (2010) compared otoferlin expression and the Ca^{2+} dependence of exocytosis at early postnatal stages of hair cell development (P1 and P7). The authors found that otoferlin expression increased from P1 to P7, but did not correlate with a switch from nonlinear Ca^{2+} dependence (power relation of ~ 2 at P1 and P7) to linear Ca^{2+} dependence, which occurs only in the mature cochlea (power relation of ~ 1 at P15). Thus, the transition from nonlinear to linear depends upon some other factor that arises during the maturation process of auditory hair cells and agrees with findings in Syt4-null mice that suggest this switch is Syt4 dependent in IHCs (Johnson et al., 2010). This is contrary to findings in vestibular hair cells that show evidence for an otoferlin-dependent linearization of the Ca^{2+} dependence (Dulon et al., 2009), further confounding the role of otoferlin in coupling calcium and exocytosis.

Beurg et al. (2010) report that IHC exocytosis is similar in otoferlin-null mice and heterozygous controls at P1, before otoferlin is normally expressed. This sug-

gests that an alternative Ca^{2+} sensor is used for exocytosis at immature IHC synapses. Because previous examination of Syt1 and Syt2 expression in IHCs has produced contradictory results (Safieddine and Wenthold, 1999; Johnson et al., 2010), Beurg et al. (2010) reexamined Syt expression and tested exocytosis in Syt-null mice. Using immunohistochemistry, they showed Syt1 and Syt2 expression in P1 hair cells. They also found Syt6 and Syt7 by RT-PCR. A parsimonious explanation is that Syts functionally replace otoferlin before P4. However, several lines of evidence from this article, as well as others, do not support this idea. Most measurements of IHC exocytosis show no difference from P0 to P3 between Syt1-, Syt2-, and Syt7-null mice and wild-type controls. Additionally, with conflicting findings as to Syt2 expression in mature hair cells (Safieddine and Wenthold, 1999; Beurg et al., 2010; Johnson et al., 2010), Beurg et al. (2010) showed no deficit in exocytosis in Syt2-null IHCs (P15–P17), which does not support the notion that Syt2 is a Ca^{2+} sensor for hair cell exocytosis after hearing onset. Overexpression of otoferlin was unable to restore synchronous vesicle fusion in Syt1-deficient chromaffin cells and hippocampal neurons. Likewise, Syt1 overexpression does not rescue exocytosis in otoferlin-deficient IHCs, which argues against a simple functional equivalence of these two proteins (T. Moser, personal communication).

The major finding of Beurg et al. (2010) is that neither Syt1, Syt2, Syt7, nor otoferlin act as Ca^{2+} sensors for exocytosis at auditory hair cell synapses during early postnatal development (before P3). Therefore, a novel Ca^{2+} sensor must fulfill this role during this period. Unanswered is why hair cells would use a different Ca^{2+} sensor. A clue may come from neuronal synapses, which use Syts to mediate synchronous release, a process that requires high Ca^{2+} concentrations, but also use a different C_2 -domain-containing Ca^{2+} sensor, Doc2b, to mediate spontaneous release, which occurs at low Ca^{2+} concentrations (Groffen et al., 2010; Pang and Südhof, 2010). Similarly, the high-fidelity hair cell synapse may require multiple Ca^{2+} sensors because the 10–30 afferent fibers contacting an individual IHC can each exhibit different spontaneous rates, acoustic thresholds, and dynamic ranges (Liberman, 1982). This suggests that individual presynaptic active zones within a particular hair cell are heterogeneous. Ca^{2+} -imaging experiments of individual hair cell synapses sup-

port this notion by revealing variations in Ca^{2+} levels across active zones in response to uniform depolarization (Frank et al., 2009). While poorly understood, heterogeneity of Ca^{2+} signaling at IHC synapses along with a need for setting threshold and dynamic range relative to spontaneous rate may highlight a unique characteristic of the hair cell synapse to match presynaptic Ca^{2+} affinities for exocytosis with postsynaptic firing properties on a per synapse basis. Thus, the evidence that Beurg et al. (2010) present of at least two, if not more, Ca^{2+} sensors in hair cells opens the possibility of adjustment of the threshold set point and dynamic range by the Ca^{2+} -sensing machinery for exocytosis. This may underlie individual synaptic properties and maximize filtering of acoustic stimuli.

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