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Tweety Homologs (TTYH) Freshly Join the Journey of Molecular Identification of the VRAC/VSOR Channel Pore

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Cell volume regulation (CVR) is fundamental to the survival and functions of animal cells under physiological and pathological conditions. Two types of chloride channels are directly activated by cell swelling and involved in CVR. One is the volume-sensitive outwardly rectifying anion channel (VSOR) [1], also called the volume-regulated anion channel (VRAC) [2], which is ubiquitously expressed and exhibits the phenotypic properties [1, 2]; and another is the maxi-anion channel (Maxi-Cl), the core component of which has recently been identified as SLCO2A1 [3]. As to the molecular identity of VSOR/VRAC (simply called VSOR hereafter), the first breakthrough was brought about by the discovery of involvements of LRRC8 members [4, 5]. In this issue of Experimental Neurobiology, Han et al. [6] makes the second breakthrough by demonstrating the involvement of Tweety homologs (TTYH) in the VSOR channel activity.

Two groups [7, 8] independently discovered the existence of functional VSOR in 1988. Since then, for a quarter of a century, its molecular identity had not been uncovered, despite much efforts of proposing and disproving many false-positive candidates, as summarized recently [9]. In 2014, two groups [4, 5] independently identified LRRC8A or SWELL1 as one of essential components of VSOR channel and reported on the same day. In addition, LRRC8C, 8D or 8E was later found to be required together with LRRC8A for functional VSOR activity [5, 10, 11]. The pore-forming roles of these LRRC8 members were suggested by discernible, though not drastic, alterations of ion selectivity by some point mutations and evidenced by successful reconstitution of anionic channel activity as well as by recent cryo-EM structure studies with purified LRRC8s, as summarized recently [12, 13]. However, it must be pointed out that several important properties (such as their cytoplasmic ATP independence, smaller single-channel conductance, and little voltage-dependent inactivation kinetics) of LRRC8-reconstituted channels are distinct from those of native VSOR channel, as summarized recently [9, 14]. More importantly, the channel reconstituted with purified LRRC8A *plus* 8D or 8E was activated by reduction of ionic strength (Γ), but not by inflation-induced membrane expansion [15]. In addition, a reduced cytoplasmic Γ was found to be required to activate the channels in HEK293 cells expressing LRRC8A alone or LRRC8A *plus* 8C [16]. In contrast, native VSOR channels can be activated by cell swelling-associated expansion of membrane infoldings [1] and inflation or membrane expansion by forcing fluid into the cells without any Γ reduction [17] (also see [12]). Thus, it is highly likely that the LRRC8 channel is not the membrane expansion-activated volume-regulatory anion channel (Me-VRAC or $VRAC_{swell}$ [6]), but the ionic strength-sensitive volume-regulatory anion channel (Is-VRAC or $VRAC_{\Gamma}$ [6]). On the other hand, it is also evident that LRRC8 members play some roles in swelling-induced activation of anion channel currents exhibiting VSOR phenotypes, because swelling-activated VSOR currents were never observed in cells lacking all five LRRC8 genes but rescued by expressing LRRC8A together with LRRC8C or 8E [5], and because coinjection of LRRC8A RNA together with LRRC8D or 8E RNA gave rise to hypotonicity-induced VSOR currents in *Xenopus* oocytes which lack all LRRC8 genes and endogenous VSOR activity [18]. However, it

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must be noted that overexpression of LRRC8A *plus* 8C failed to increase swelling-induced VSOR currents above the endogenous level of VSOR currents in wild-type HEK293 and HCT116 cells [5]. Furthermore, cisplatin-resistant KCP-4 cells which exhibited distinctly smaller VSOR activity showed similar gene expression levels of all LRRC8 members to those in the parental KB cells as well as in other three different human epithelial cells [9, 19]. Moreover, overexpression of LRRC8A *plus* 8D or 8E in KCP-4 cells failed to restore VSOR activity up to the level in its parental KB cells [19]. Taken together, it appears that some as-yet-unidentified pore-related component other than LRRC8 members is required for VSOR channel activity. Thus, through the upsurge of LRRC8 studies for the past five years, the field has been faced with the questions: What is the missing component for the VSOR pore and how LRRC8 members are precisely involved in the VSOR activation mechanism? Now, C. Justin Lee's group [6] reports a pioneering research in astrocytes to answer these questions.

By surprise, Han et al. [6] observed that *Lrrc8a* shRNA and/or *Lrrc8c* shRNA never suppressed the peak amplitude, though slowed the activation time course, of hypotonicity-induced VSOR currents in mouse astrocytes and human HEK293 cells when exposed to Tris-Cl-rich extracellular and intracellular solutions that do not contain cation channel-permeable small cations, Na^+ and K^+, but with only a trace amount of Cs^+. On the other hand, they also observed that *Lrrc8a*-shRNA suppressed VSOR currents in HEK293 cells under NaCl-rich conditions but not in HEK293 cells overexpressing TTYH1 and AQP4 genes. These observations are in contrast to the previous observations made by other groups in astrocytes, as follows. Treatment with *Lrrc8a*-shRNA and *Lrrc8a*-siRNA nearly abolished hypotonicity-induced VSOR currents in rat astrocytes under NMDG-Cl-rich and CsCl-rich conditions, respectively [20, 21], and VSOR currents activated by hypotonic stimulation or by glutamate-induced GPCR stimulation were largely, though not completely, abolished in astrocytes derived from astrocyte-specific *Lrrc8a* (*Swell1*) knockout mice under intracellular CsCl-rich and extracellular NMDG-Cl-rich conditions [22]. In place of LRRC8 members, Han et al. [6] paid attention to an involvement of Tweety homologs, TTYH1, TTYH2 and TTYH3, because TTYH1/2/3 genes are highly expressed in astrocytes [23], and because TTYH1 was previously proposed as a candidate molecule for another volume-activated Maxi-Cl channel [24], though later disproved [9]. Actually, *Ttyh1/2/3*-shRNA virtually abolished hypotonicity-induced VSOR currents in mouse astrocytes in culture and in hippocampal CA1 slices under Tris-Cl-rich conditions [6]. Furthermore, Han et al. [6] strongly suggested TTYH1 as a VSOR pore-forming component, based on the following two observations. First, overexpression of the charge-neutralizing R165A mutant of TTYH1 or the R164A mutant of TTYH2 largely suppressed VSOR currents with reducing Cl^- permeability in HEK293 cells under Tris-Cl-rich conditions. Second, VSOR currents in HEK293 cells overexpressing R165C-TTYH1 was substantially sensitive to MTSES under Tris-Cl-rich conditions. In light of these results, as concluded by Han et al. [6], it is likely that the TTYH family represents *bona fide* Me-VRAC channel in astrocytes.

Like the last breakthrough with LRRC8, this time starting with Tweety homologs has opened novel enigmas to be unraveled in future studies. Why is astrocytic VSOR activity not sensitive to gene silencing of LRRC8A under Tris-Cl-rich conditions, although it was sensitive to the gene silencing under NMDG-Cl-rich conditions free of small cations? How is CVR exactly attained by the indirect interaction between LRRC8 and TTYH members (such as the model proposed in Fig. 12 [6]) in astrocytes? What about an involvement of the direct protein-protein interaction between LRRC8 and TTYH members in the CVR mechanism in astrocytes? What/how about an involvement of any TTYH member in swelling-independent VSOR activation (see [14, 25]) induced by GPCR stimulation and by reactive oxygen species (ROS)? What about an involvement of any TTYH member in VSOR activity in non-astrocytic cells? What is an alternative molecule involved in Me-VRAC activity in non-astrocytic cells, such as human epithelial cells, which do not significantly express TTYH genes? Lastly, to precisely verify the pore-forming roles of LRRC8A/C/D/E in Is-VRAC and of TTYH1/2/3 in Me-VRAC, drastic alterations in the anion selectivity Eisenman's sequence and in the anion/cation permeability ratio must be observed with some charge-modifying (especially charge-reversing) mutations. At last, the VRAC/VSOR journey still continues with Tweety's surprising debut in the realm of molecular identification of the pore, following the recent LRRC8 upsurge.

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