LETTER

Validity of pHluorin-tagged GluA2 as a reporter for AMPA receptor surface expression and endocytosis

The recent paper by Rathje et al. (1) questions the interpretation of previous studies using super ecliptic pHluorin-GluA2 (SEP-GluA2) to monitor AMPA receptor surface expression and particularly its use as a reporter of endocytosis following NMDA or AMPA receptor stimulation. The concerns raised are based on two main observations: (*i*) that application of the agonists NMDA or AMPA leads to intracellular acidification and (*ii*) that the acid (pH 6) wash, which is routinely used in these experiments to identify surface receptors, can also cause intracellular acidification, leading to misidentification of surface and intracellular receptors.

i. In the first paper to use SEP-GluA2 as a marker for surface-expressed AMPA receptors in living cells, extensive control experiments demonstrated that application of NMDA did not change intracellular pH, as measured by the pH-sensitive dye 2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein (BCECF) (2). This intracellular acidification assay is undoubtedly a critical control and the reasons for the differences reported by Ashby et al. and Rathje et al. remain to be fully determined. We note, however, that Rathje et al. use a greatly reduced Mg^{2+} concentration (300 μM) during NMDA application compared with previous studies (3–5). Indeed, in our hands, stimulation of dispersed hippocampal neurons with NMDA in media containing 300 μM Mg^{2+} caused a rapid and sustained intracellular acidification, an effect that we did not observe in our standard media containing 1.5 mM Mg^{2+}.

ii. With respect to the acid wash, although many of the experiments recapitulate published work (5), Rathje et al. use different protocols to those previously optimized. Because the acid wash is designed to specifically identify surface receptors, Ashby et al. (5) stressed the importance of primarily analyzing plasma membrane and avoiding analysis of the whole soma, stating "We attribute the intracellular fluorescence mainly to SEP-GluA2 in the endoplasmic reticulum, which is less acidic than other secretory organelles and is prominent in the cell body." Crucially, the low pH wash should be limited to seconds rather than minutes to report surface fluorescence and avoid intracellular acidification (5).

Rathje et al. expose their neurons to low pH for what appears to be 15 min. Under these very long exposures to low pH, it is unsurprising that intracellular acidification occurs. In contrast, brief acid washes using rapid perfusion and confocal imaging rates, combined with analysis of plasma membrane localized fluorescence rather intracellular regions, effectively eliminate nearly all intracellular SEP signal. Using these simple parameters, SEP-GluA2 fluorescence is a reliable and robust marker of AMPA receptor endocytosis.

Overall, the message from Rathje et al. is the need for caution in the acquisition, analysis, and interpretation of SEP-GluA2 data. We agree entirely. However, when used appropriately, SEP-GluA2 is an extremely valuable tool for monitoring the properties and dynamics of AMPA receptor surface diffusion, endocytosis, and recycling in living neurons.

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Author contributions: K.A.W., M.C.A., and J.M.H. designed research; K.A.W. performed research; K.A.W., M.C.A., and J.M.H. analyzed data; and K.A.W., M.C.A., and J.M.H. wrote the paper.

The authors declare no conflict of interest.

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www.pnas.org/cgi/doi/10.1073/pnas.1319322111