Reply to Wilkinson et al.: Concerning the use of pHluorin-tagged GluA2 as a reporter for NMDA-induced AMPA receptor recycling

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As pointed out in the letter by Wilkinson et al. (1), we question in our recent paper by Rathje et al. (2) the use of pHluorin-GluA2 (pH-GluA2) as a reporter of NMDA- and AMPA-induced endocytosis because application of the agonists NMDA and AMPA results in intracellular acidification of hippocampal neurons. This conclusion rests on two important key observations: (*i*) the pHluorin response to NMDA treatment was almost intact after removal of surface-exposed pHluorin by thrombin cleavage (figure 1 of Rathje et al.) (2) and (*ii*) NMDA stimulation reduces fluorescence of both ER-retained pH-GluA2 and cytosolic pHluorin, in agreement with intracellular acidification, which was further supported by measurements using the pH-sensitive dye SNARF-1 (figures 2 and 3 of Rathje et al.) (2).

Intracellular acidification in response to NMDA has previously been reported (3) and was observed with our stimulation protocol (20 μM NMDA, 300 μM Mg^{2+}, 50 μM glycine, and 1 μM TTX), using both cytosolic pHluorin and the pH-sensitive dye SNARF-1 (2). However, Wilkinson et al. (1) describe in their letter that no intracellular acidification was observed using a different stimulation protocol (50 μM NMDA, 1.5 mM Mg^{2+}, and no glycine or TTX). We have now performed experiments addressing the differences in the stimulation protocol. We find that the 1.5 mM Mg^{2+} protocol induced robust, although less dramatic, acidification than the 300 μM Mg^{2+} protocol, as measured using cytosolic pHluorin, indicating that even under these conditions, pH-GluA2 is not a valid reporter of NMDA- and AMPA-induced AMPA receptor recycling.

Ashby et al. (4) described a control experiment identifying surface-exposed pHluorin by a brief acid wash. In our recent paper, we described experiments with prolonged acid wash (figure S4 of Rathje et al.) (2), which suggested that the neuronal plasma membrane is permeable to protons. These experiments were merely performed to stress the point that this approach should be limited to brief washes, which indeed requires rapid perfusion and confocal imaging rates, as described by Ashby et al. (4). However, it is important to note that identification of surface-exposed pHluorin by a brief acid wash does not necessarily mean that a subsequent NMDA-induced decrease in the pHluorin signal originates from this surface-exposed fraction. On the contrary, our experiments with thrombin protease cleavage of surface pHluorin suggest that the decreased pHluorin signal mainly originates from intracellular receptor pools. Thus, we still question the use of pH-GluA2 as a valuable tool for monitoring NMDA- and AMPA-induced AMPA receptor endocytosis.

1 Wilkinson KA, Ashby MC, Henley JM (2014) Validity of pHluorin-tagged GluA2 as a reporter for AMPA receptor surface expression and endocytosis. *Proc Natl Acad Sci USA* 111:E304.
2 Rathje M, et al. (2013) AMPA receptor pHluorin-GluA2 reports NMDA receptor-induced intracellular acidification in hippocampal neurons. *Proc Natl Acad Sci USA* 110(35):14426–14431.
3 Irwin RP, Lin SZ, Long RT, Paul SM (1994) N-methyl-D-aspartate induces a rapid, reversible, and calcium-dependent intracellular acidosis in cultured fetal rat hippocampal neurons. *J Neurosci* 14(3 Pt 1):1352–1357.
4 Ashby MC, et al. (2004) Removal of AMPA receptors (AMPARs) from synapses is preceded by transient endocytosis of extrasynaptic AMPARs. *J Neurosci* 24(22):5172–5176.

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The authors declare no conflict of interest.

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