Supplemental Figures

Figure S1. Confocal imaging showing co-localization of GluR2 with NKA. Cultured cortical neurons were immunostained sequentially with antibodies against GluR2 (red) and NKA α1 (green). An optical slice acquired from confocal single scan revealed co-localization of NKA hotspots with AMPAR puncta (left). The boxed area was shown in separate channels for clarity (right).

Figure S2. Ouabain dose dependency and NKA inhibition by potassium-free solution. (A) Cortical neurons were incubated for 1 hr with ouabain of varied concentrations as indicated. A reduction in GluR1 and GluR2/3 was induced by as low as 0.1 µM ouabain, and progressively more dramatic changes were observed along with higher ouabain concentration. (B) Cortical neurons were incubated with ACSF, or ACSF containing 0 KCl for 1 hr. Inhibition of NKA by K-free treatment dramatically reduced the abundance of GluR1 and GluR2/3. Western membrane was reprobed with anti-tubulin to show equal loading.

Figure S3. Agrin as an NKA inhibitor induces membrane depolarization and AMPAR degradation. (A and B) Whole-cell recording was performed on cultured cortical neurons under current-clamp configuration. Application of ouabain (50 µM) and Agrin 4,8 (medium of agrin-expressing HEK cells, 1:1000) to the recording chamber induced a rapid membrane depolarization accompanied with high frequency firing of action potentials. (C and D) Cortical neurons were incubated with agrin 0,0 (1:1000) and agrin 4,8 (1:1000) for 1 hr. Cell lysates were separated with SDS-PAGE and probed with GluR1 antibodies. Treatment of both agrin isoforms induced marked reduction in GluR1 levels (n=3, p<0.05 compared to control).
Figure S4. Ouabain induces rapid accumulation of intracellular sodium. 2-wk-old cortical neurons were incubated with ouabain (50 µM) in ACSF for varied periods of time. CoroNa Green-AM (0.5 mM) was then supplemented to the coverslips for 10 min and imaged immediately under a fluorescence microscope. CoroNa showed a gradual increase in intensity with ouabain treatment. Top panel are typical images at different time points of treatment; the lower panel shows the average increase in CoroNa intensity (n=20 cells).

Figure S5. Intracellular sodium accumulation reduces AMPAR abundance. Cortical neurons were incubated with veratridine (20 µM) in the presence of glutamate receptor antagonist cocktail containing CNQX (20 µM), APV (50 µM) and MCPG (500 µM) to block AMPARs/KARs, NMDARs and mGluRs, respectively. 1 hr treatment with veratridine induced a reduction in GluR1 abundance compared to antagonists alone (control), indicating the sufficiency of sodium accumulation in AMPAR removal.

Figure S6. Ouabain treatment does not affect NKA surface expression. Following 1 hr ouabain (50 µM) treatment, plasma membrane proteins of cortical neurons were isolated by surface biotinylation. While surface GluR1 was reduced by the treatment, membrane NKA levels were not affected, suggesting that AMPARs do not co-traffic with NKA during internalization.

Figure S7. Recovery time course of ouabain effect. Following 1 hr ouabain treatment, neurons were lysed immediately (0 min) or incubated in regular culture medium to recover for 30, 60, and 120 min, respectively, prior to lysis. Only a modest recovery in GluR1 amount was observed 1-2 hrs after treatment.
Figure S8. Inhibition of tyrosine kinase activity does not affect ouabain effect. Neurons were treated with a tyrosine kinase-specific inhibitor genistein (100 µM) alone or together with ouabain (50 µM) for 1 hr. In the presence of genistein, ouabain still induced about 30% reduction in GluR1 amount (n=4, * P<0.05, t test).