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Brief Communications

PICK1 and Phosphorylation of the Glutamate Receptor 2 (GluR2) AMPA Receptor Subunit Regulates GluR2 Recycling after NMDA Receptor-Induced Internalization

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Changes in surface trafficking of AMPA receptors play an important role in synaptic plasticity. Phosphorylation of the C terminus of the AMPA receptor (AMPAR) subunit glutamate receptor 2 (GluR2) and the binding of GluR2 to the PDZ [postsynaptic density-95/Discs large/zona occludens-1]-domain containing protein, protein interacting with protein kinase C (PICK1), have been proposed to play an important role in NMDA receptor dependent internalization of GluR2. However, the fate of internalized GluR2 after NMDA receptor (NMDAR) activation is still unclear. Both recycling and degradation of GluR2 after the activation of NMDAR have been reported. Here, we used a pH-sensitive green fluorescent protein variant, pHluorin, tagged to the N terminus of GluR2 (pH-GluR2) to study the dynamic internalization and recycling of GluR2 after NMDAR activation. Using fluorescence recovery after photobleach (FRAP), we directly demonstrate that internalized pH-GluR2 subunits recycle back to the cell surface after NMDAR activation. We further demonstrate that changing the phosphorylation state of the S880 residue at the C terminus of GluR2 does not affect NMDAR-dependent GluR2 internalization, but alters the recycling of GluR2 after NMDAR activation. In addition, mutation of the *N*-ethylmaleimide-sensitive fusion protein (NSF) binding site in the pH-GluR2 slows receptor recycling. Finally, neurons lacking PICK1 display normal NMDAR dependent GluR2 internalization compared with wild-type neurons, but demonstrate accelerated GluR2 recycling after NMDAR activation. These results indicate that phosphorylation of GluR2 S880 and NSF and PICK1 binding to GluR2 dynamically regulate GluR2 recycling.

Key words: AMPA receptor; endocytosis; phosphorylation; PICK1; NSF; FRAP

Introduction

AMPA receptors (AMPARs) and NMDA receptors (NMDARs) are the major excitatory neurotransmitter receptors in the CNS. AMPARs mediate the majority of fast excitatory synaptic transmission while NMDARs play an essential role in the induction of certain forms of synaptic plasticity (Hollmann and Heinemann, 1994; Dingledine et al., 1999). The regulation of the dynamic membrane trafficking of AMPARs plays a critical role in controlling the levels of synaptic AMPARs and in the expression of several forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD). A variety of studies have demonstrated that phosphorylation of AMPARs and AMPAR interacting proteins dynamically regulate receptor trafficking and the expression of LTP and LTD (Song and Huganir, 2002). Several studies have shown that the AMPAR interacting protein PICK1 regulates the surface expression of glutamate receptor 2 (GluR2) (Perez et al., 2001; Terashima et al., 2004) and disrupting GluR2–PICK1 interactions blocks the expression of LTD (Xia et al., 2000; Iwakura et al., 2001; Kim et al., 2001; Chung et al., 2003). In contrast, the AMPAR interacting protein *N*-ethylmaleimide-sensitive fusion protein (NSF) increases the surface expression of GluR2 (Huang et al., 2005). Disrupting NSF binding to GluR2 decreases AMPAR mediated synaptic transmission (Nishimune et al., 1998; Song et al., 1998) and the surface expression of GluR2 (Noel et al., 1999). NSF has been proposed to regulate GluR2 surface expression by disassembling GluR2–PICK1 complexes (Hanley et al., 2002). Interestingly, phosphorylation of serine 880 (S880) within the C terminus of the GluR2 subunit differentially regulates the interaction of GluR2 with two postsynaptic density-95/Discs large/zona occludens-1 (PDZ)-domain containing proteins, glutamate receptor interacting protein 1/2 (GRIP1/2) and PICK1, and modulates the membrane trafficking of GluR2 and the expression of LTD (Xia et al., 2000; Chung et al., 2003; Seidenman et al., 2003; Steinberg et al., 2006). Together, these data indicate that interaction of PICK1 with GluR2 either promotes internalization or inhibits recycling of GluR2 and retains GluR2 intracellularly. The precise role of PICK1 in regulating GluR2 trafficking, however, remains unclear.

To examine the regulation of AMPAR membrane trafficking in more detail we have used the pH-sensitive green fluorescent protein, pHluorin (Miesenbock et al., 1998), fused to the N terminus of the GluR2 (pH-GluR2) to study AMPAR membrane

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trafficking with high temporal resolution (Ashby et al., 2004; Kopec et al., 2006). This construct can be used to monitor the endocytosis/exocytosis of GluR2 in response to NMDAR activation. By combining imaging pH-GluR2 with fluorescence recovery after photobleach (FRAP), we demonstrated that after NMDAR activation, pH-GluR2 recycled back to the surface. We further demonstrated that altering the phosphorylation states of GluR2 S880 residue as well as disrupting PICK1 binding to GluR2 did not impair NMDAR induced GluR2 internalization. Instead, alterations in this phosphorylation site resulted in changes in the rate of recycling of pH-GluR2 after NMDAR activation. Finally, we showed that in PICK1 knock-out hippocampal cultures, GluR2 recycling after NMDAR activation was accelerated although GluR2 internalization in response to NMDAR activation remained unchanged. These results indicate that phosphorylation of GluR2 S880, which differentially regulated PICK1 binding to GluR2, also regulates GluR2 recycling to the plasma membrane.

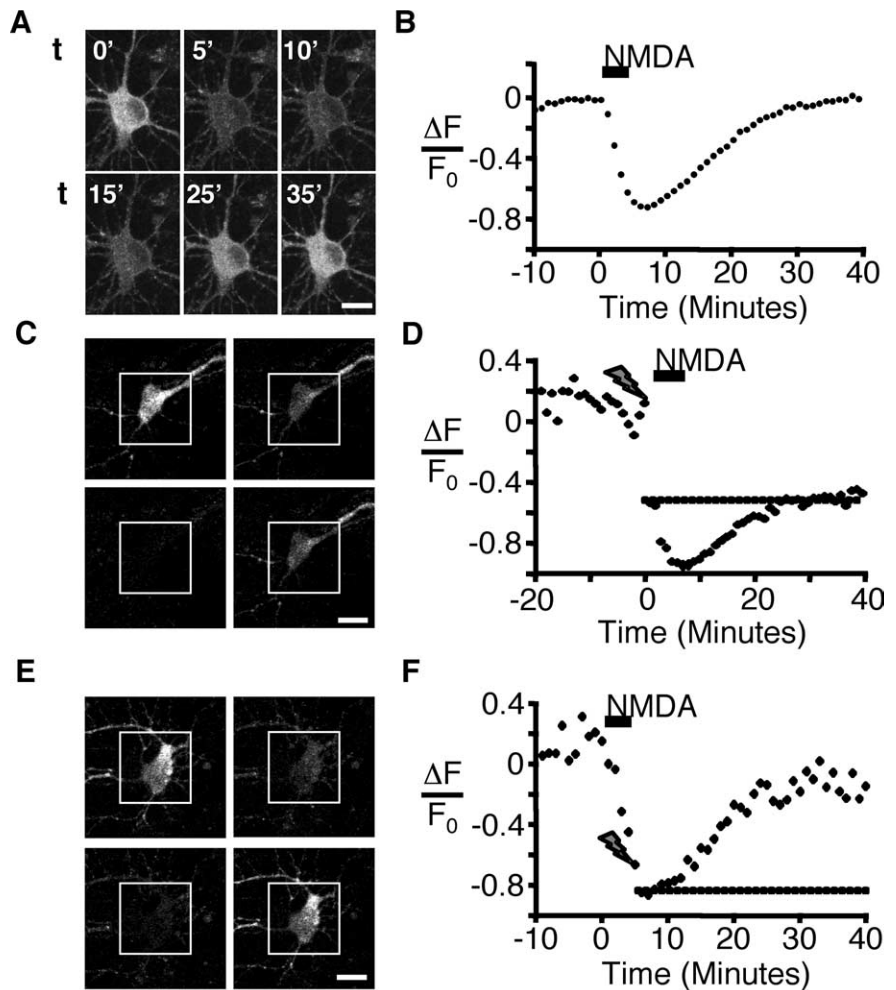

Figure 1. pH-GluR2 is internalized following NMDAR activation and recycles to the cell surface. ***A***, Representative images of a hippocampal neuron subjected to NMDA perfusion/washout cycle show that NMDA induces loss of significant amount of observed pH-GluR2 fluorescence; washout of NMDA resulted in full return of pH-GluR2 fluorescence over time. ***B***, Time trace of pH-GluR2 fluorescence change in response to 5-min NMDA treatment for the experiment presented in ***A***. ***C***, A hippocampal neuron expressing pH-GluR2 shown (top left). pH-GluR2 on the surface of the cell soma was photobleached immediately before NMDA perfusion, resulting in loss of the majority of pH-GluR2 fluorescence in this somatic area (top right). NMDA perfusion for 5 min induces pH-GluR2 internalization (bottom left). NMDA washout results in full recovery of pH-GluR2 fluorescence in dendritic surface (unbleached area) and recovery of pH-GluR2 fluorescence to postbleached level in somatic surface (bleached area, white square). ***D***, Time trace of fluorescence change in somatic (bleached) surface for the experiment presented in ***C***. ***E***, A hippocampal neuron expressing pH-GluR2 is shown (top left). The pH-GluR2 relocates to endosomal compartments after 5-min NMDA perfusion, resulting in loss of the majority of pH-GluR2 fluorescence (top right). The same photobleach protocol used in ***C*** was then applied to the surface of somatic area immediately before NMDA washout, resulting in a small additional loss of pH-GluR2 fluorescence in the somatic area (bottom left). NMDA washout results in complete recovery of pH-GluR2 fluorescence on the dendritic surface (unbleached area) and almost complete recovery of pH-GluR2 fluorescence on the somatic surface (bleached area, white square). ***F***, Time trace of fluorescence change in somatic (bleached) surface for the experiment presented in ***E***. Scale bars: ***A***, ***C***, ***E***, 10 μm.

Materials and Methods

All chemicals were obtained from Fisher Scientific (Pittsburgh, PA) unless otherwise specified. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA).

Super Ecliptic pHluorin coding sequence was amplified by PCR and inserted into the N terminus of GluR2 after signal peptide. To generate C-terminal point mutants of pH-GluR2, the C terminus of GluR2 S880E, K882A (Chung et al., 2003), and ΔNSF (Steinberg et al., 2004) were exchanged with that of pH-GluR2 by subcloning. DNA sequences were verified by automated DNA sequencing in the Johns Hopkins University School of Medicine DNA sequencing Core facility. For details about hippocampal neuronal cultures and transfection and imaging and data analysis see the supplemental Methods (available at www.jneurosci.org as supplemental material).

Results

Use of pHluorin tagged GluR2 fusion proteins to analyze the membrane trafficking of the AMPAR GluR2 subunit after NMDAR activation

Ecliptic pHluorin has the distinctive feature that it is fluorescent at pH ~7.4 and nonfluorescent at pH <6.0 under 488 nm excitation. The N terminus of GluR2 is exposed to either the extracellular space (pH ~7.4) or the lumen of endosomes (pH <6.0) when the receptors reside on the plasma membrane or endosomes, respectively. The pH-GluR2 construct was engineered such that pHluorin was inserted into the N terminus of GluR2 after the signal peptide. The change of pHluorin fluorescence can then be used as an indicator of receptor redistribution between plasma membrane and endosomal compartments after NMDAR activation (Ashby et al., 2004). When pH-GluR2 was expressed in hippocampal neurons, the majority of pH-GluR2 fluorescence originated from surface-exposed pH-GluR2 proteins and was sensitive to rapid changes in extracellular pH (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). Moreover, perfusion of NMDA for 5 min, which is known to induce rapid internalization of GluR2, induced a significant loss of pH-GluR2 fluorescence. Interestingly, washout of NMDA resulted in full recovery of fluorescence within 30 min (Fig. 1*A*,*B*, supplemental movie 1, available at www.jneurosci.org as supplemental material). This process could be repeated and the pH-GluR2 demonstrated similar kinetics of loss/recovery of fluorescence over several cycles in both somatic and dendritic areas (supplemental Fig. 1*B*, available at www.jneurosci.org as supple-

mental material). However, we did not observe significant change in fluorescence of dendritic pH-GluR2 punta (most likely synaptic clusters of GluR2) in response to 5 min NMDAR activation over a 1 h period of recording. The fluorescence loss/recovery of pH-GluR2 induced by NMDA perfusion/washout was fully blocked by APV, a specific NMDAR antagonist (supplemental Fig. 1*C*, available at www.jneurosci.org as supplemental material), suggesting that the change of pHluorin fluorescence required NMDAR activation. In addition, the change of pHluorin fluorescence in response to NMDA perfusion was highly sensitive to extracellular Ca^{2+} concentration (supplemental Fig. 1*D*, available at www.jneurosci.org as supplemental material), indicating that the change of fluorescence after NMDAR activation required calcium entry and was not likely caused by changes in pH values in either extracellular solutions or intracellular environments. Furthermore, this change of pH-GluR2 fluorescence is fully blocked by two membrane-permeable dynamin inhibitory peptides, but not a membrane permeable control peptide (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (Brebner et al., 2005). Together, these data demonstrate that the fluorescence loss during NMDA perfusion represents NMDAR induced GluR2 internalization and the fluorescence recovery after NMDA washout represents insertion of pHluorin GluR2 into the plasma membrane.

To determine whether the inserted GluR2 after NMDAR induced internalization was caused by *de novo* insertion of new receptors or recycling of the internalized receptors, we combined imaging of pH-GluR2 during NMDA perfusion/washout with FRAP. We reasoned that if the inserted receptors were from preexisting intracellular pools, NMDA perfusion/washout after photobleach should result in full recovery of pH-GluR2 fluorescence, assuming that the nonfluorescent intracellular pools cannot be photobleached. Conversely, if the inserted receptors were those that were originally present on the surface and internalized by NMDAR activation, NMDA perfusion/washout after photobleach should result in recovery of pH-GluR2 fluorescence only to the postphotobleach level, because of the fact that the reinserted receptors would carry a photobleached and nonfluorescent form of pHluorin. A hippocampal neuron expressing pH-GluR2 before photobleach is shown in Figure 1*C* (top left). The surface of the soma was chosen for photobleach to minimize possible contribution of lateral diffusion to the fluorescence recovery after photobleach. After photobleach, a significant proportion of pH-GluR2 fluorescence was eliminated (Fig. 1*C*, top right). Subsequent perfusion of NMDA for 5 min induced GluR2 internalization in both the somatic and dendritic areas (Fig. 1*C*, bottom left). pH-GluR2 fluorescence in a unbleached area (dendritic surface) fully recovered to baseline level 30 min after NMDA washout (Fig. 1*C*, bottom right). However, pH-GluR2 fluorescence in the photobleached area (somatic surface) only recovered to the postphotobleach level (Fig. 1*C*, bottom right, *D*). This result suggests that after NMDAR activation, the inserted pH-GluR2 was originated from recycled receptors that were originally present on the surface. However, an alternative explanation could be that the lack of full recovery of fluorescence was attributable to either photodamage to the cellular machinery responsible for receptor trafficking or photobleach of intracellular invisible receptor pools. To address this issue, a second FRAP experiment was performed in which the pH-GluR2 was first relocated to intracellular pools by the activation of NMDA receptors and photobleach was then performed immediately before NMDA washout. We reasoned that if the photobleach protocol caused photodamage to the receptor trafficking machinery or photobleach of intracellular invisible pH-GluR2 pools, the photobleached area should also demonstrate a lack of recovery of pH-GluR2 fluorescence. Conversely, if the photobleach caused no damage to the trafficking machinery, a full recovery of fluorescence after NMDA washout should be observed. A hippocampal neuron expressing pH-GluR2 is shown in Figure 1*E* (top left). After stimulation with NMDA for 5 min the majority of pH-GluR2 fluorescence disappeared consistent with relocation of receptors to intracellular compartments (Fig. 1*E*, top right). The same photobleach protocol was then performed (Fig. 1*E*, bottom left). We observed a small amount of additional fluorescence loss in the bleached area, which is attributable to photobleach of the remaining surface pH-GluR2. After photobleach, fluorescence of pH-GluR2 fully recovered on the dendritic surface (nonbleached) and almost fully recovered on the somatic surface (photobleached) area (Fig. 1*E*, bottom right, *F*). The slight reduction in the fluorescence recovery on the somatic surface was likely caused by photobleach of remaining surface pH-GluR2 fluorescence during our photobleach protocol. Together, these two FRAP experiments demonstrate that the inserted pH-GluR2 after NMDAR activation is derived from recycled receptors that were internalized in response to NMDAR activation.

Phosphorylation state of GluR2 S880 regulates GluR2 recycling

The S880 residue in the C terminus of GluR2 has been shown to be a target site for PKC phosphorylation *in vitro* and *in vivo* (Chung et al., 2000). In addition, we showed that after NMDAR activation, both PKC activity and phosphorylation level of GluR2 S880 residue were transiently increased and returned to baseline after NMDA washout (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), suggesting that NMDAR activation can regulate phosphorylation state of GluR2 S880 through activation of PKC pathway. A variety of data have shown that changes in the phosphorylation state of S880 residue regulates activity dependent GluR2 trafficking. However, it has been difficult to analyze this in detail using either biotinylation or immunostaining approaches. To examine the effect of changes in the S880 residue phosphorylation state on the dynamics of GluR2 membrane trafficking in response to NMDAR activation, we generated two pH-GluR2 point mutants. One mutant, pH-GluR2 S880E, should mimic phosphorylation of this residue whereas the other mutant, pH-GluR2 K882A, should inhibit PKC phosphorylation of S880 without affecting PDZ domain interactions (Chung et al., 2000). Hippocampal neurons expressing either pH-GluR2 S880E or K882A are shown in Figure 2*A*. Perfusion of NMDA for 5 min induced internalization of pH-GluR2, pH-GluR2 S880E, and pH-GluR2 K882A at levels of 73.8 ± 1.6% ($n = 19$), 71.0 ± 3.4% ($n = 5$), and 68.0% + 3.0% ($n = 4$), respectively (Fig. 2*B*,*C*). There is no statistical difference in NMDAR activation induced GluR2 internalization between pH-GluR2 wt and S880E ($p = 0.443$, *t* test) or pH-GluR2 wt and K882A ($p = 0.144$, *t* test). However, interestingly, reinsertion of pH-GluR2 displayed a $T_{1/2}$ (see Materials and Methods) of 10.3 ± 0.4 min ($n = 19$) after NMDA washout, whereas the reinsertion of pH-GluR2 S880E was significantly prolonged ($T_{1/2} = 16.8 \pm 1.4$ min; $n = 5$; $p < 0.001$, *t* test) (Fig. 2*B*,*C*). In contrast, pH-GluR2 K882A showed accelerated reinsertion after NMDA washout ($T_{1/2} = 6.3 \pm 0.8$ min; $n = 4$; $p < 0.001$, *t* test) (Fig. 2*B*,*C*). These results indicated that the change in the phosphorylation state of GluR2 S880 is not critical for NMDAR activation induced GluR2 internalization, but instead regulates the recycling of GluR2 after NMDAR activation.

NSF affects GluR2 recycling after NMDAR activation

NSF has been shown to dissociate the complex of GluR2 and PICK1 (Hanley et al., 2002), and this activity of NSF has been shown to facilitate synaptic targeting of GluR2-containing AMPARs (Steinberg et al., 2004; Gardner et al., 2005). To test whether NSF facilitates recycling of GluR2 after NMDAR activation, a pH-GluR2 mutant that eliminated the NSF binding site (pH-GluR2 ΔNSF: V848L, A849T, P852T point mutation) (Steinberg et al., 2004) was analyzed in the assay. A hippocampal neuron expressing pH-GluR2 ΔNSF is shown in Figure 2*A* (bottom). Perfusion of NMDA for 5 min induced 75.0 ± 2.8% internalization of surface pH-GluR2 ΔNSF ($n = 6$), similar to that of pH-GluR2 ($p = 0.731$, *t* test). However, the recycling of pH-GluR2 ΔNSF was significantly prolonged after NMDA washout ($T_{1/2} = 14.2 \pm 1.1$ min; $n = 6$; $p < 0.001$, *t* test) (Fig. 2*B*, bottom, *C*). This suggests that NSF facilitates the recycling of GluR2 after NMDAR activation.

***PICK1* knock-out results in accelerated GluR2 recycling process**

To test the role of PICK1 in regulating GluR2 recycling after NMDAR activation, we examined pH-GluR2 internalization/recycling in *PICK1* knock-out neurons (Gardner et al., 2005). Hippocampal neurons were derived from postnatal day 0 (P0) wild-type or *PICK1* knock-out mice and transfected with pH-GluR2 (Fig. 3*A*). NMDA perfusion for 5 min induced 61.8 ± 3.2% of pH-GluR2 internalization in PICK1 knock-out neurons ($n = 5$), and 63.0 ± 5.5% in neurons derived from wild-type mice ($n = 5$; $p = 0.855$, *t* test). This result suggests that PICK1 is not required for NMDA induced GluR2 internalization in hippocampal neurons (Fig. 3*B*,*C*). However, after NMDA washout, *PICK1* knock-out neurons demonstrated significant acceleration of GluR2 recycling ($T_{1/2} = 4.8 \pm 0.4$ min; $n = 5$) compared with control neurons ($T_{1/2} = 11.6 + 0.9$ min; $n = 5$; $p < 0.001$, *t* test) (Fig. 3*B*,*C*). This result strongly suggests that, at least in hippocampal neurons, PICK1 retains internalized GluR2 after NMDAR activation and inhibits GluR2 recycling back to the plasma membrane.

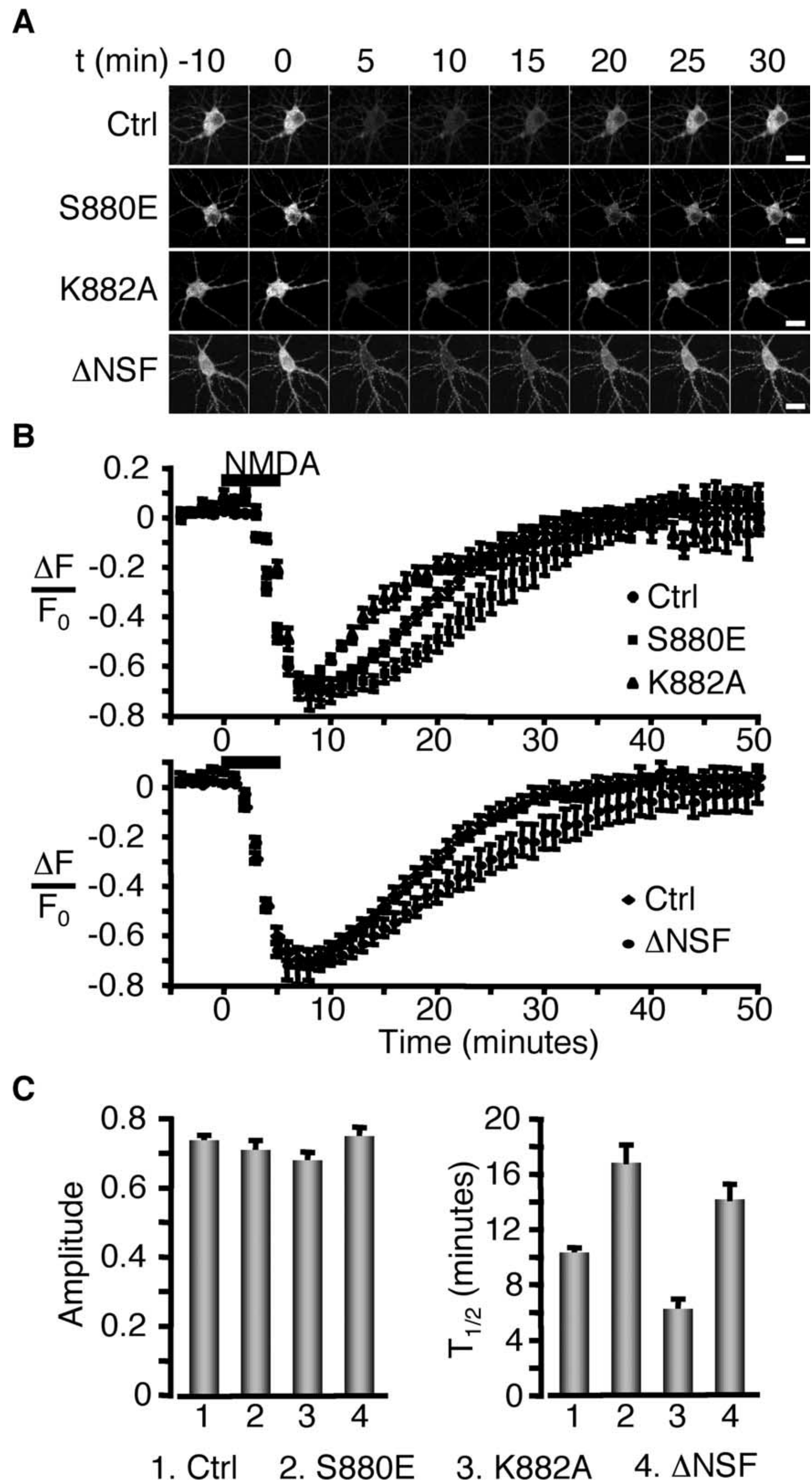

Figure 2. Modulating GluR2 S880 phosphorylation state as well as NSF binding to GluR2 alters GluR2 recycling process. ***A***, Selective images of pH-GluR2 wt, S880E, K882A, and ΔNSF during NMDA perfusion/washout experiments. ***B***, Average fluorescence time course for pH-GluR2 wt, S880E, K882A, and ΔNSF mutants during NMDA perfusion/washout experiments. ***C***, Histograms of pHluorin fluorescence change amplitude in response to NMDA and $T_{1/2}$ after NMDA washout. Scale bars: 10 μm.

Discussion

Previous studies have demonstrated that the regulation of the surface membrane trafficking of AMPA receptors plays an important role in synaptic plasticity (Song and Huganir, 2002; Bredt and Nicoll, 2003). In addition, recycling endosomes is suggested to supply additional AMPA receptors during LTP induction, emphasizing the importance of the regulation of AMPAR recycling (Park et al., 2004). However, the molecular mechanisms regulating AMPAR recycling remain elusive. This is in part because of the lack of tempo-

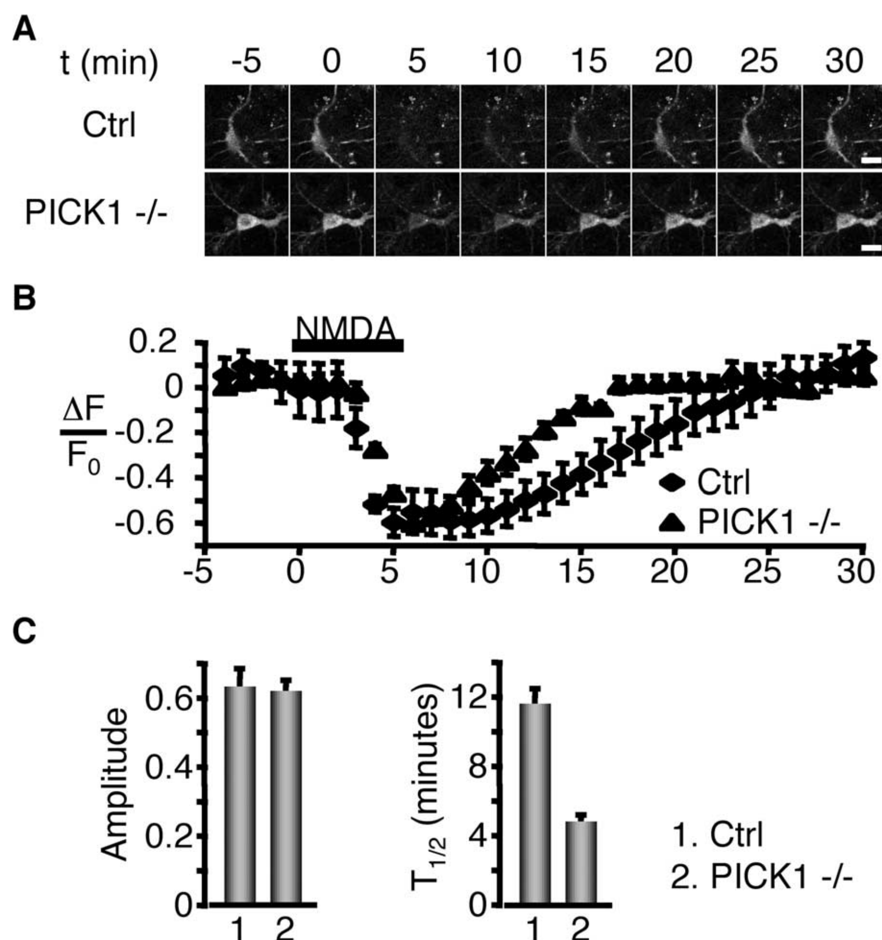

Figure 3. Neurons derived from PICK1 knock-out mice displays altered GluR2 recycling. ***A***, Selective images of pH-GluR2 from wild-type and PICK1 knock-out hippocampal neurons during NMDA perfusion/washout experiments. ***B***, Average fluorescence time course of pH-GluR2 from wild-type and PICK1 knock-out hippocampal neurons during NMDA perfusion/washout experiments. ***C***, Histograms of pHluorin fluorescence change amplitude in response to NMDA and $T_{1/2}$ after NMDA washout. Scale bars: 10 μm.

ral resolution of existing assays for receptor recycling. Here, we used receptors tagged with ecliptic pHluorin to examine AMPAR recycling process quantitatively and with fine temporal resolution. By combining imaging pH-GluR2 and FRAP, we demonstrated that GluR2 recycles back to the surface after NMDAR activation, in agreement with early findings using cell surface biotinylation assays (Ehlers, 2000). Using this approach, we further showed that the phosphorylation state of GluR2 S880 residue as well as PICK1 binding to GluR2 regulates the rate of GluR2 recycling after NMDAR activation.

The PDZ motif at the end of GluR2 C terminus binds both PICK1 and GRIP. In addition, S880 residue within this motif is also a PKC phosphorylation site (Chung et al., 2000, 2003; Xia et al., 2000; Kim et al., 2001). In the absence of S880 phosphorylation, GluR2 preferentially binds GRIP (Chung et al., 2000; Kim et al., 2001). Phosphorylation of S880 results in disruption of GRIP binding to GluR2 without affecting PICK1 binding (Chung et al., 2000, 2003; Kim et al., 2001). Thus, it has been suggested that the phosphorylation state of GluR2 S880 modulates GluR2 surface trafficking by regulation of GluR2 interaction with its binding partners (Song and Huganir, 2002). However, it has been difficult to examine the effect of GluR2 phosphorylation on the dynamics of receptor trafficking. In addition, it has not been clear whether PICK1 plays a direct role in GluR2 endocytosis or if it only retains internalized GluR2 intracellularly, or both. By monitoring pH-GluR2 fluorescence changes during NMDA perfusion/washout, we found that the magnitude of internalization induced by NMDAR activation was not significantly different between pH-GluR2 wt and S880E, K882A, or ΔNSF constructs, as well as in neurons from both *PICK1* knock-out mice and wild-type littermates. This suggests that neither PICK1 nor the phosphorylation of GluR2 S880 residue play a critical role in NMDAR induced GluR2 internalization process, at least in hippocampal neurons. In contrast, our data directly demonstrate that PICK1 regulates GluR2 recycling after NMDAR activation. pH-GluR2 K882A, a GluR2 mutant that cannot be phosphorylated by PKC and preferentially binds GRIP, displays accelerated recycling after NMDA washout. However, pH-GluR2 S880E, which mimics S880 phosphorylation and binds PICK1 but not GRIP, displays delayed recycling. In addition, pH-GluR2 ΔNSF also demonstrates prolonged recycling. Because NSF has been shown to disassemble GluR2-PICK1 complex (Hanley et al., 2002), NSF is likely to exert its role in regulating GluR2 recycling through dissociating PICK1 from GluR2. Together, these data indicate that PICK1 favors retention of internalized GluR2 in recycling pools. Consistently, pH-GluR2 displays accelerated recycling after NMDAR activation in neurons derived from PICK1 knock-out mice. These data strongly suggest that phosphorylation of GluR2 S880 and the binding of PICK1 to GluR2 regulate the recycling of internalized GluR2. PICK1-GluR2 interaction has been shown to be important for cerebellar and hippocampal LTD, two forms of long-lasting synaptic depression (Chung et al., 2000, 2003; Daw et al., 2000; Xia et al., 2000; Iwakura et al., 2001; Kim et al., 2001), as well as calcium-permeable AMPAR plasticity at cerebellar parallel fiber stellar cell synapse (Gardner et al., 2005; Liu and Cull-Candy, 2005). The binding of PICK1 to GluR2 is likely to regulate intracellular pools of GluR2 receptors in recycling endosomes that can be differentially regulated in different cell types in response to distinct signaling pathways.

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