Published in final edited form as:

Biochem Soc Trans. 2003 August ; 31(Pt 4): 880–884.

Real-time imaging of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor) movements in neurons

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Abstract

The mechanisms that regulate α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) synthesis, transport, targeting and surface expression are of fundamental importance for fast excitatory neurotransmission and synaptic plasticity in the mammalian central nervous system. It has become apparent that these control processes involve complex sets of protein–protein interactions and many of the proteins responsible have been identified. We have been working to visualize AMPAR movement in living neurons in order to investigate the effects of blocking protein interactions. Here we outline the approaches used and the results obtained thus far.

Keywords

α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor); confocal microscopy; green fluorescent protein; neuron; real-time imaging; receptor trafficking

Introduction

α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) are responsible for most fast excitatory neurotransmission in the mammalian central nervous system and they are crucial for the processes of synaptic stabilization and plasticity [1–3]. AMPARs are multimeric assemblies of the subunits GluR1–GluR4 [4]. Each subunit comprises $\approx$ 900 amino acids and has a molecular mass of $\approx$ 105 kDa. The N-terminus is extracellular, there are three membrane-spanning and one re-entrant loop domains and the C-terminus is intracellular [5,6] (Figure 1). Under basal conditions it appears that 50–70% of AMPARs are intracellular [7,8], with a significant proportion being localized inside dendrites [9,10].

Transport and targeting of AMPARs within neurons

Neurons are highly compartmentalized cells that can possess thousands of synapses each containing multiple receptors with potentially differing subunit compositions. There are several routes by which receptors may be trafficked in neurons including long-distance diffusion in the plasma membrane and/or transport of mRNAs within the cell and local synthesis of proteins close to their destination [11]. However, perhaps the predominant method is likely to be intracellular vesicular transport to the vicinity of their target site

(synapse), followed by exocytosis [12] and then lateral diffusion in the lipid bilayer and anchoring at their target destination [13]. Consistent with this latter model, bi-directional vesicle transport has been reported in both axons and dendrites [14]. Vesicles are driven by the molecular motor proteins kinesin and dynein, responsible for movements towards opposite ends of the microtubules. Thus polarized vesicle trafficking is likely to require specific interactions between domain-specific motor(s) and vesicle cargo. Indeed, it has been shown recently that inhibition of dynein or kinesin by binding of monoclonal antibodies substantially reduced synaptic AMPAR-mediated, but not *N*-methyl-D-aspartate (NMDA)-receptor-mediated, responses [15]. Furthermore, both NMDA receptors [16,17] and AMPARs [18] undergo cargo-selective polarized vesicle transport via dendrite-specific kinesin motors. There remains, however, much that is unclear. For example, targeting of proteins to specific sites is likely to require either address motifs in the cargo proteins themselves or some kind of a recognition domain allowing passing vesicles to be captured and retained at relevant cellular locations. The nature and identities of these motifs and the molecular interactions involved have not yet been identified.

Dynamic regulation of functional synaptic AMPARs

AMPAR recycling under basal conditions

Until a few years ago synaptic AMPARs were thought to be relatively stable under basal conditions with a constitutive turnover of surface-expressed receptors of the order of hours to days [8,19,20]. However, it has now been established that GluR2-containing AMPARs undergo rapid recycling under basal conditions with a half-life at the surface in the order of a few minutes. This rapid constitutive turnover is dependent on the direct non-PDZ-domain-mediated interaction between GluR2 and *N*-ethylmaleimide-sensitive fusion protein (NSF) [15,21-27] and has had major implications for understanding the cellular processes underlying synaptic plasticity (for reviews see [28-30]). The principle of rapid NSF-dependent recycling has also been extended to G-protein-coupled receptors via β -arrestins [31] and γ -aminobutyric acid type A (GABA_A) receptors via GABARAP (GABA receptor associated protein) [32], suggesting that this may be an important general regulatory synaptic mechanism.

Recently it has also been shown that AP2, a clathrin adaptor complex important for endocytosis, associates with a region of GluR2 that overlaps the NSF-binding site and it has been suggested that, whereas NSF interactions are required to maintain synaptic AMPARs but are not involved in NMDA-receptor-mediated internalization and long-term depression, AP2 is involved specifically in NMDA-receptor-induced AMPAR endocytosis and is required for hippocampal long-term depression [33].

Activity-dependent endo- and exocytosis of AMPARs

The synaptic expression of functional AMPARs is highly regulated both during development and by neuronal activity. For example, an important characteristic of glutamatergic synapses is that they can exhibit NMDA receptor responses in the absence of functional AMPARs and, because of the magnesium block of NMDA receptors at resting membrane potentials, these postsynaptic membranes are functionally inactive or 'silent' [34,35]. Subsequent immunolocalization studies have shown synapses that contain NMDA receptors but no AMPARs [36-38] and have demonstrated that AMPARs can be recruited to the synapse within minutes by either spontaneous or stimulated NMDA-receptor activation [39-41]. The 'unsilencing' of these synapses by the rapid insertion of functional AMPARs is likely to be a key determinant for NMDA-receptor-dependent synaptic plasticity and neuronal development [42].

The regulation of AMPAR surface expression is undoubtedly a complex process that requires multiple protein interactions. Several proteins have been identified that interact selectively with individual AMPAR subunits and the best, but still incompletely, characterized protein interactors include the PDZ-domain-containing proteins GRIP and PICK1 for GluR2 subunit and SAP97 for the GluR1 subunit. These interactors appear to be particularly important for activity-dependent changes in AMPAR and also the related kainate receptor synaptic expression (e.g. [43]; for reviews see [44-47]).

Several studies have focused on identifying the molecular basis of AMPAR recycling at the postsynaptic membrane. The internalization of AMPARs can be triggered by NMDA-receptor activation and is mediated by the formation of clathrin-coated pits [48]. Disruption of clathrin-mediated endocytosis, for example by biochemical inhibition of pit formation or overexpression of a dominant-negative form of dynamin, effectively blocks the internalization of AMPARs in a GluR2-dependent manner [49,50]. Furthermore, a proportion of AMPAR turnover appears to be directly regulated by synaptic activity, with removal from the synaptic membrane occurring in a ligand-dependent manner. Increasing synaptic activity using picrotoxin, a GABA-receptor antagonist, has been shown to cause a decrease in the number of surface-expressed AMPARs and in the size of AMPAR clusters in cultured neurons [51]. Conversely, decreasing synaptic activity using tetrodotoxin or AMPAR antagonists causes an increase in synaptic AMPAR levels [50]. Exogenous application of glutamate or AMPA causes internalization and redistribution of AMPARs but probably only in a subset of synapses as mEPSCs (miniature excitatory postsynaptic currents) decrease in frequency but not size [51-53]. This can occur in both an NMDA-receptor-dependent and -independent fashion, with differential implications for the fate of internalized receptors. AMPARs internalized in response to NMDA-receptor activation are rapidly recycled and reinserted into the synaptic membrane; in contrast, treatment with AMPA causes the internalized receptors to be targeted to protein-degradation pathways [50,54].

Protein kinase pathways and AMPAR regulation

Protein phosphorylation events are known to play a central role in AMPAR surface expression and to modify existing functional synaptic channel properties in a complex but highly adaptable manner. For example, long-term potentiation (LTP) or calcium/calmodulin-dependent protein kinase II (CaMKII) activation has been reported to drive GluR1-containing AMPARs to synapses [55]. Protein kinase A (PKA)-mediated phosphorylation of GluR1 in combination with CaMKII has also been suggested to directly control the synaptic incorporation of AMPARs [56]. More recently it has been reported that unlike LTP in mature hippocampus, LTP in immature animals (less than 9 days old) requires PKA but not CaMKII mechanisms [57]. This highlights that there are developmental changes in neuronal plasticity and that kinase sensitivity is linked to these changes.

The situation is further complicated by the observations that the precise nature and functional consequence of GluR1 phosphorylation is also dependent on the stimulation history of the synapse. One hypothesis is that induction of long-term depression in naive synapses dephosphorylates the major PKA site, whereas in previously potentiated synapses the major CaMKII site is dephosphorylated. Correspondingly, LTP induction in naive synapses increases phosphorylation of the CaMKII site while LTP induction in previously depressed synapses increases phosphorylation of the PKA site [58].

Related studies have shown that the small GTPase 'molecular switches' Ras and Rap are also involved in the control of AMPAR trafficking and in the postsynaptic signalling underlying synaptic plasticity. Ras mediates activity-evoked increases in GluR1/GluR4 containing AMPAR surface expression at synapses via a pathway that requires p42/44

mitogen-activated protein kinase activation. In contrast, Rap mediates NMDA-receptor-dependent removal of synaptic GluR2/3-containing AMPARs via a pathway that involves p38 mitogen-activated protein kinase [59]. Thus Ras and Rap, as well as proteins that regulate them, such as SynGAP [60,61], may add another level of control to AMPAR surface expression at synapses.

Visualizing AMPAR translocation and surface expression

The use of green fluorescent protein (GFP)-tagged AMPAR subunits expressed in neurons with viral vectors has had a significant impact on the field. In hippocampal slice cultures it has been reported that tetanic synaptic stimulation causes the rapid NMDA-receptor-dependent delivery of GFP-GluR1 into dendritic spines in hippocampal slice preparations [62]. GFP-labelled subunits have been visualized in combination with electrophysiological tagging, where the channel rectification properties of recombinant AMPARs comprising specific subunits are altered by point mutations, e.g. GluR2(R586Q)-GFP. Using these approaches it has been proposed that there are differential targeting mechanisms for AMPARs comprising either GluR1/GluR2 or GluR2/GluR3 subunit assemblies [55]. More specifically, the hypothesis is that GluR1/GluR2 receptors are added to synapses during plasticity via a process that requires interactions between GluR1 and group I PDZ domain proteins [55]. In this model CaMKII and LTP drive synaptic expression of GluR1-containing AMPARs. In contrast, GluR2/GluR3 receptors replace existing synaptic receptors in a constitutive manner dependent on interactions of GluR2 with NSF and group II PDZ domain proteins [63].

In our laboratory, current experiments are aimed at investigating two of the basic questions which remain concerning AMPAR trafficking: (i) how AMPARs are directed to the correct locations in the neuron as and when required and (ii) what are the temporal and spatial characteristics of AMPAR surface expression?

Intracellular movement of GFP-GluR1

To begin to address the dynamics of how AMPARs are translocated around the cell interior, we are directly visualizing the intracellular transport of AMPARs using Sindbis virus-infected GFP-GluR1 in dispersed cultures of hippocampal neurons. Consistent with previous immunocytochemical data for native GluR1 from our cell cultures [10], we observe that intracellular GFP-tagged AMPARs are widely distributed throughout the somatodendritic compartment. Some GFP-GluR1-containing receptors are surface expressed with a synaptic distribution indistinguishable from native AMPARs. Furthermore, native and exogenously expressed subunits can be co-immunoprecipitated, indicating that they co-assemble into receptor complexes.

In dynamic, near-real-time imaging experiments we observed no evidence for intracellular clusters of receptors either in the soma or the dendrites suggesting the relatively free translocation of AMPARs in the soma and dendrites of neurons. We went on to assess the rates and directions of AMPAR movement using fluorescence recovery after photobleach (FRAP) and fluorescence loss in photobleach (FLIP) techniques. Our data show that AMPARs are transported in dendrites at rates comparable with fast axonal transport and that they move in a predominantly, but not exclusively, proximal to distal anterograde direction. Over the time course of our experiments AMPAR transport in dendrites was not altered by activation or inhibition of ionotropic glutamate receptors or by addition of glycine, a protocol known to induce LTP in dispersed cultures [41]. From these initial experiments we propose that the intracellular transport of GFP-GluR1-containing AMPARs is not activity-regulated, but rather that various subunit combinations of the receptor complex are likely to

be translocated widely throughout the soma and dendrites. Synapses may then ‘capture’ these passing receptors as and when required.

Use of pH-sensitive GFP (pHluorin) to monitor surface expression of GluR2

A limitation of studies using GFP to study the location of plasma-membrane receptors is that it is inherently difficult to distinguish between proteins in the cell membrane and those which are nearby but still inside the cell. In an attempt to address this, random mutation studies of wild-type GFP led to the discovery of GFP variants, termed pHluorins, whose fluorescence is highly sensitive to changes in pH. In particular, ecliptic pHluorin (EP) gradually decreases in fluorescence intensity as the pH is lowered, such that the fluorescence signal is negligible at pH values <6.0 [64]. Since the membrane compartments which deliver proteins to the cell surface are relatively acidic, this means that, upon delivery to the cell surface, where it is exposed to neutral pH, EP exhibits a rapid increase in fluorescence. To make use of these properties we have constructed, characterized and used a GluR2 AMPAR subunit fused at the extracellular N-terminus with pHluorin (pHluorin–GluR2; the pHluorin DNA was a gift from Jim Rothman, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.).

When expressed in HEK-293 cells the pHluorin–GluR2 showed only low-level fluorescence since most expression is confined to the intracellular compartment. However, when cells were treated with NH_4Cl to increase the intracellular pH, there was a robust and reversible increase in signal. The pHluorin–GluR2 co-assembled with wild-type GluR1 to form fully functional heteromeric AMPA receptors.

We used adenovirus to express pHluorin–GluR2 in cultured hippocampal neurons. Although the pHluorin–GluR2 is expressed throughout the cells, the receptors at the cell surface are readily distinguishable because of their higher fluorescence and sensitivity to extracellular pH changes. Surface pHluorin–GluR2 largely co-localized with endogenous GluR1 and with the synaptic markers synaptophysin and FM4–64, indicating that the protein is delivered to functional synapses. Initial experiments have indicated that K^+ depolarization of the neurons can trigger activity-dependent changes in surface expression. Furthermore, activation of NMDA receptors via a protocol reported to induce NMDA-receptor-dependent LTP (200 μM glycine, 2 min) caused a rapid, robust and long-lasting increase in fluorescence due to increased surface-expressed pHluorin–GluR2-containing AMPARs at new and existing membrane sites on the soma and the dendrites. These results suggest that pHluorin–GluR2 provides a powerful new tool for the study of the dynamics of AMPAR surface expression in neurons and may be useful for investigating the mechanisms underlying synaptic plasticity.

Abbreviations used

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA	AMPA receptor
GFP	green fluorescent protein
NMDA	<i>N</i> -methyl-d-aspartate
NSF	<i>N</i> -ethylmaleimide-sensitive fusion protein
GABA_A	γ -aminobutyric acid type A
LTP	long-term potentiation
CaMKII	calcium/calmodulin-dependent protein kinase II

PKA protein kinase A

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Figure 1.
Schematic representation of the membrane topology of an AMPAR subunit tagged at the N-terminus with green fluorescent protein-based fluorophore