Endosomal sorting of AMPA receptors in hippocampal neurons

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Abstract
An important mechanism for the regulation of excitatory synaptic transmission in the hippocampus involves tight control of AMPAR [AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor] trafficking to alter the number or subtype of synaptic receptors. This is achieved via the multiple stages of the endosomal system. AMPARs constitutively cycle through early endosomes and recycling endosomes to maintain synaptic receptor numbers. However, on induction of synaptic plasticity, subtle alterations are made to this cycle by the action of specific AMPAR-interacting proteins and also via a number of additional proteins that regulate endosomal sorting more generally. During long-term depression, receptors are diverted to late endosomes and lysosomes rather than recycling back to the plasma membrane, hence reducing the number of receptors at the synapse. The increased number of synaptic AMPARs after induction of LTP (long-term potentiation) originates from the recycling compartment. In addition, transient changes in subunit composition may arise as a result of retention of AMPAR subtypes within the endosome during LTP. Aberrant trafficking after pathological insults such as oxygen/glucose deprivation or mechanical trauma also involves alterations in synaptic AMPAR subunit composition, leading to calcium influx that ultimately results in cell death.

Introduction
AMPA receptors [AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors] mediate most of the fast excitatory synaptic transmission in the brain. Therefore the regulation of synaptic AMPARs is profoundly important to brain function. In particular, synaptic plasticity, which is thought to underlie learning and memory, involves the modulation of synaptic strength by regulating AMPAR number and subunit composition. LTD (long-term depression) and LTP (long-term potentiation) are well-studied phenomena that represent such long-lasting changes in synaptic transmission, and are brought about largely by the regulated trafficking of AMPARs to and from the synaptic plasma membrane [1–3]. Early studies suggested that LTD involves the internalization of AMPARs by endocytosis to reduce the number of receptors at the synapse [4,5], and LTP involves the insertion of additional AMPARs by exocytosis to increase synaptic AMPAR numbers [6,7]. Although these basic principles are still considered correct, more recent studies have demonstrated that the situation is actually much more elaborate, involving complex trafficking stages that implicate endosomal sorting. In the present review, I will discuss some of these aspects of AMPAR trafficking that are central to synaptic plasticity in the hippocampus and that also play an important role in aberrant synaptic transmission in neuronal pathology in this brain region.

Basic trafficking mechanisms
In addition to regulated trafficking during LTD/LTP, AMPARs undergo constant rounds of constitutive endocytosis/exocytosis in such a way that internalized receptors are recycled back to the plasma membrane (Figure 1). Changes in AMPARs expressed at the synapse are therefore brought about by adjustments to this constitutively dynamic system [8].

Receptor recycling occurs via the multiple compartments of the endosomal system, which is not specific to neurons, but is used by many cell types to regulate the trafficking of numerous cell surface receptors [9]. After internalization, endocytic vesicles carrying membrane-bound receptors are targeted to the early endosome, from where they are sorted to either a recycling endosome or a late endosome. Proteins that are sorted to the late endosome are usually subsequently targeted for lysosomal degradation. Receptors targeted to recycling endosomes can then be returned to the plasma membrane. AMPARs have been found co-localized with all of these intracellular membrane compartments [10,11].

LTD
Although endocytosis of AMPARs from the plasma membrane is required for LTD expression [5], the fate of internalized receptors after internalization is also a crucial factor. It has been suggested that during chemically induced LTD [brief bath application of NMDA (N-methyl-d-aspartate)], AMPARs are diverted from the recycling pathway, and sorted
Figure 1 | Endosomal trafficking pathways followed by AMPARs

(1) Constitutive trafficking (dotted arrow): AMPARs are internalized from the plasma membrane by clathrin-mediated endocytosis, and traffic in endocytic vesicles to early endosomes. Via the action of NSF on GluR2, possibly by disrupting PICK1–GluR2 interactions that may restrict AMPAR recycling, AMPARs are targeted to the recycling endosome. This sorting step also requires the NEEP21–GRIP–GluR2 complex. From the recycling endosome, receptors traffic to the plasma membrane.

(2) LTD (dark grey arrow): AMPAR endocytosis is enhanced by LTD stimuli, and the internalized receptors traffic to the early endosome, a process involving Rab5 and probably PICK1. LTD stimuli modulate GluR2 interactions with PICK1 and NSF, resulting in the targeting of AMPARs to the late endosome, instead of the recycling compartment. Receptors are subsequently degraded in the lysosome. (3) LTP (light grey arrow): AMPARs present in the recycling system are trafficked through the recycling endosome, involving the action of Rab11 and Rme1, to the plasma membrane. GluR2 may be retained in the recycling or even early endosomal compartment possibly via interaction with PICK1, resulting in a transient incorporation of GluR2-lacking receptors at the synaptic plasma membrane. There may also be some contribution via a Rab8 pathway from the TGN to the recycling endosome.

instead to the late endosome and ultimately the lysosome [11]. Therefore the number of receptors that are recycled back to the synaptic plasma membrane is reduced, leading to a reduction in synaptic strength. An important event in this process must therefore be at the level of the early endosome, where the fate of the internalized AMPAR is determined. After NMDA application, endogenous AMPARs exhibit a transient increase in co-localization with early endosomal markers EEA1 (early endosome antigen 1) and Rab5 [10]. Although this increase could in theory reflect a reduction in traffic out of the early endosome, it is most likely to be the result of an increase in AMPAR endocytosis, since direct imaging of the removal of recombinant AMPAR subunits from the cell surface has demonstrated that chemical LTD enhances AMPAR endocytosis [12,13].

Rab5 is an important regulator of the function of early endosomes [14]. Expression of a Rab5 dominant-negative mutant in hippocampal neurons blocks LTD, but not constitutive AMPAR trafficking, demonstrating an active role for Rab5 in LTD [15]. Overexpression of wild-type Rab5 mimics LTD, supporting this observation. However, it should be noted that Rab5 plays a role in several stages of early endosome function, including the formation of clathrin-coated endocytic vesicles and their fusion with early endosomes, as well as the motility of early endosomes and their fusion with each other [14].

The molecular mechanism that underlies the decision-making process at the level of the early endosome may involve AMPAR accessory proteins such as NSF (N-ethylmaleimide-sensitive fusion protein), PICK1 (protein that interacts with protein C-kinase 1) and GRIP (glutamate receptor-interacting protein). In a study using recombinant AMPAR subunits expressed in hippocampal neurons, mutations in GluR2 (glutamate receptor) 2 at the NSF-binding site redirected receptors to lysosomes for degradation even in the absence of a chemical LTD stimulus, suggesting that a
function of the NSF–GluR2 interaction is to keep AMPARs in the recycling system and prevent them from entering the degradation pathway [11]. The above-mentioned study also investigated the role of the GluR2 PDZ ligand in endosomal sorting. Surprisingly, GluR2 mutated at this site showed the same pattern of progression through the endosomal pathway as the wild-type [11]. This result appears to be in contrast with other reports, which implicate the PDZ proteins PICK1 and GRIP in regulating AMPAR recycling.

The precise role of GRIP (and its close relative ABP (AMPA-binding protein)) in regulating AMPAR trafficking is still far from clear. An early study demonstrated that disrupting GRIP–GluR2 interactions with a blocking peptide inhibits LTD, suggesting that GRIP restricts AMPARs from the recycling pool after internalization [16]. More recently, it has been shown that GRIP functions as a scaffold protein, binding (among many other proteins) both GluR2 and NEEP21 (neuronal enriched endosomal protein of 21 kDa), forming a complex that is required for recycling of GluR2-containing AMPARs. Disruption of this complex resulted in reduced levels of surface GluR2, increased GluR2 accumulation in early endosomes and also increased targeting to lysosomal compartments [17,18]. This suggests that the GRIP–NEEP21 interaction is required for internalized AMPARs to exit early endosomes and traffic back to the plasma membrane, presumably via recycling endosomes. This appears to be contradictory to the paper by Daw et al. [16], although the role of the GRIP–NEEP21 interaction in LTD was not tested. Further work is necessary to define the role of GRIP in AMPAR trafficking.

PICK1 is a BAR (Bin/amphiphysin/Rvs) domain protein, consistent with a role in vesicle trafficking [19]. A study using pHluorin-tagged GluR2 homomers indicated that in the absence of PICK1 binding, these receptors were recycled more rapidly back to the cell surface after NMDA-induced internalization, suggesting that PICK1 may function to restrict recycling [13]. However, another report indicated that PICK1 interactions can actually facilitate AMPAR recycling to the plasma membrane [20]. PICK1 has been found to co-localize with Rab11, which is a marker for recycling endosomes, and also with Rab5, a marker for endocytic vesicles and early endosomes, but not with the late endosomal marker Rab7 [21,22]. Co-localization with the recycling endosomal marker Rab11 was greater than with the early endosomal marker Rab5. These observations are consistent with a role for PICK1 in regulating the trafficking of GluR2-containing AMPARs through early and recycling endosomal compartments. A role for PICK1 in restricting GluR2 recycling and NSF in facilitating recycling is consistent with the observation that the ATPase activity of NSF functions to disrupt GluR2–PICK1 interactions [23].

Since these GluR2-interacting proteins are implicated in synaptically induced LTD [16,24–26], an attractive mechanism is that LTD induction stimuli regulate GluR2 interactions with NSF, GRIP and PICK1 to divert AMPARs away from recycling, and towards the degradative pathway. This is supported by the observation that NMDAR (NMDA receptor) activation leads to reduced total levels of AMPAR subunits in hippocampal neurons, an effect that is blocked by inhibitors of lysosomal but not proteasomal degradation [11].

The regulatory mechanisms discussed here for LTD occur at the endosomal level, although it should be noted that in many cases, the results are also consistent with control of endocytosis at the plasma membrane. For example, Rab5 is a key regulator of multiple stages of endocytosis. It is involved in clathrin-coated vesicle formation, fusion between early endosomes and endosomal motility [14]. Therefore experiments defining a role for Rab5 in LTD do not specify precisely whether endocytosis or motility of early endosomes is being manipulated. Similarly, the localization of PICK1 to Rab5- as well as Rab11-positive compartments [21,22] suggests that it may function to regulate endocytosis from the plasma membrane as well as subsequent recycling back to the cell surface. Perhaps most importantly, although the mechanism discussed so far has implicated NMDAR activation in regulating sorting to recycling or late endosomes, strong evidence also exists for NMDA-dependent regulation of AMPAR endocytosis from the plasma membrane [12,13].

**LTP**

LTP involves the insertion of additional AMPARs at the synaptic plasma membrane [7]. It has been shown that GluR1-containing AMPARs inserted at the synaptic plasma membrane in response to LTP-inducing stimuli originate from the plasma membrane and therefore must have passed through recycling endosomes [27]. This suggests that LTP, as well as LTD, involves manipulation of the recycling pathway. Rab11 and Rme1 are both required for normal endosomal recycling. Disrupting the recycling pathway by expressing dominant-negative mutants of these proteins blocked AMPAR delivery during LTP, further supporting this hypothesis [27]. Perhaps the most intriguing observation in the study by Park et al. [27] was that LTP stimuli promoted recycling not only of AMPARs, but also of transferrin receptors, suggesting that LTP involves a global regulation of the recycling system, rather than a specific regulation of AMPAR trafficking through recycling endosomes [27]. Little is known about GluR1 protein interactions that regulate trafficking through recycling compartments. SAP97 (synapse-associated protein 97) and 4.1N have both been shown to play a role in GluR1 trafficking during LTP, but have not been implicated in the recycling pathway. The observation that LTP enhances recycling non-specifically suggests an absence of control by specific AMPAR interactors.

Whereas LTP has been shown to enhance non-specific receptor recycling, LTD does not appear to have a similar converse effect by inhibiting general recycling. In contrast, LTD appears to regulate AMPAR recycling by tight control of GluR2-interacting proteins, which presumably function to sort receptors for differential targeting to endosomal compartments.
Similar studies using mutant Rab proteins to disrupt trafficking through specific endosomal compartments have suggested that Rab8, as well as Rab11, is required for AMPAR delivery during LTP [28,29]. Although Rab8 is found associated with recycling endosomes, its function is not as well defined as other members of the family. It has been suggested that in non-neuronal cells, Rab8 specifically regulates traffic of proteins through the recycling endosome that have originated from the TGN (trans-Golgi network) rather than from the plasma membrane [30]. This may contradict the idea that AMPARs inserted at the synapse during LTP originate from the plasma membrane [27]. Surprisingly, a Rab8 dominant-negative mutant not only blocks LTP, but also inhibits constitutive AMPAR trafficking [28], the latter of which presumably utilizes the recycling pathway to a greater extent than the TGN pathway. The precise route of intercompartmental trafficking regulated by Rab8 may require further investigation before these results for AMPAR trafficking can be fully interpreted.

**Subunit specificity**

A number of studies have made use of recombinantly expressed AMPAR subunits to examine the effect of plasticity-inducing stimuli on receptor trafficking [11–13,15,28,31–33]. Although this approach provides important indicators about the mechanisms that underlie the subunit-specific regulation of AMPAR trafficking, overexpressed subunits may not fully represent the true situation for endogenous receptors. Based on this approach, it appears that the GluR2 subunit and protein interactions with the GluR2 C-terminal domain are largely responsible for regulating AMPAR trafficking during chemically and synthetically induced forms of LTD [11,33]. A distinct function for GluR1 in LTD has not been described, apart from the well-established observation that dephosphorylation of GluR1 at Ser845 correlates with AMPAR internalization [10]. The precise role of this dephosphorylation event in regulating receptor trafficking is still unknown.

In an early model for the subunit-specific regulation of AMPAR trafficking during LTP, GluR1 drives the insertion of GluR1–GluR2 heteromers at the synapse, and the subsequent maintenance of the potentiated levels of synaptic AMPARs is mediated by constitutive internalization and recycling of GluR2–GluR3 heteromers, which replace the newly inserted GluR1-containing receptors [31,32]. An important aspect of this model is that of ‘slot proteins’, which are theoretical proteins (or protein complexes), yet to be assigned actual protein identities, but proposed to be PSD-95 (postsynaptic density 95)/TARPs (transmembrane AMPAR regulatory proteins). Additional slot proteins are inserted into the synaptic plasma membrane along with AMPARs during LTP and maintain the position for an AMPAR complex during rounds of constitutive trafficking [31,34].

More recent studies have required modifications to this model. Since GluR1-containing receptors inserted during LTP originate from the plasma membrane [27], GluR1, as well as GluR2/3, must be found in recycling endosomes. This implies that a significant proportion of constitutively recycling AMPARs contain GluR1 subunits. Furthermore, since LTP stimuli enhance the recycling of transferrin receptors (and by implication, any membrane-bound receptor found in recycling endosomes) [27], it is reasonable to assume that LTP will also drive the synaptic insertion of GluR2–GluR3 receptors from the recycling system. This idea is not consistent with studies suggesting that the trafficking of GluR2–GluR3 receptors is unaffected by LTP stimuli [31,32].

A further development of the model, which is still somewhat controversial, is based on the observation that after LTP induction, a transient switch in synaptic AMPAR subunit composition occurs, such that a greater proportion of GluR2-lacking AMPAR is expressed at the synapse for approx. 20 min ([35,36] but see [37]). Since the GluR2 subunit confers calcium impermeability on AMPAR complexes, GluR2-lacking AMPARs are calcium-permeable and therefore the functional consequence of this switch is a transient increase in AMPAR-mediated calcium influx [38].

It is postulated that this calcium signalling is important for initiating long-term changes in synaptic transmission that underlie LTP. The simplest mechanistic explanation for this switch is that GluR2-lacking AMPARs are selectively inserted at the synapse. Since the vast majority of AMPARs contain GluR2 subunits [39,40], an important question is how are GluR2-lacking AMPARs selectively targeted to the synaptic plasma membrane in response to LTP stimuli? One possibility is that a mechanism exists to specifically retain GluR2-containing AMPARs in the endosomal system, while allowing GluR2-lacking receptors to traffic to the synapse. PICK1 is a likely candidate as a mediator of this mechanism, since it has been suggested to restrict GluR2 recycling [13], and it is also required for LTP expression [26].

An equally important question is how are the transiently expressed GluR2-lacking AMPARs later replaced by GluR2-containing receptors? This could simply involve a removal of the GluR2-specific retention mechanism, so that normal recycling of all AMPARs in the appropriate endosomal compartments (most of which contain GluR2) is resumed. Alternatively, a mechanism may exist to actively promote forward traffic of GluR2-containing AMPARs at some stage after LTP induction.

This model would require a small supply of GluR2-lacking AMPARs to be continually present in the recycling pool, such that under basal conditions, they would represent an insignificant fraction of synaptic receptors. However, after LTP induction, a proportion of GluR2-containing receptors would be retained in the endosomal system, whereas constitutive trafficking continues, leaving GluR2-lacking AMPARs to replace GluR2-containing AMPAR. The total number of AMPAR positions at the synapse is maintained by slot proteins. Experimentally, this would be observed as insertion of GluR1 [7,31,32].
Pathological situations
The transient increase in GluR2-lacking AMPARs observed during the early stages of LTP expression [35] may reflect a mechanism that occurs after OGD (oxygen and glucose deprivation) of hippocampal neurons, which is an in vitro model for ischaemia [41]. OGD for 30 min results in a reduction of synaptic GluR2 (but not GluR1), which leads to cell death 30 h later [41,42]. In contrast with LTP, where the switch lasts just 20 min, OGD appears to induce a more permanent (>24 h) shift to GluR2-lacking AMPARs [41]. Although this long-lasting effect may partly be a result of reduced GluR2 subunit mRNA expression, which can be detected after approx. 12 h post-insult [43], the changes observed before this time reflect a bona fide trafficking event. The clear difference is that during LTP, the calcium influx via transient synaptic targeting of GluR2-lacking calcium-permeable AMPARs contributes to signalling pathways that enhance synaptic transmission, whereas during OGD, the sustained synaptic targeting of GluR2-lacking AMPARs results in toxic levels of calcium influx. In both cases, the switch is NMDAR dependent, and is also PICK1 dependent, suggesting that the mechanism is similar to the one proposed above for the subunit switch during LTP [42].

A similar mechanism regulates hippocampal AMPARs in response to a mechanical stretch, which is an in vitro model for traumatic brain injury [44]. Intracellular retention of GluR2 is enhanced by mild stretch and activation of NMDAR in a PICK1-dependent mechanism, resulting in increased GluR2-lacking AMPARs at the neuronal surface, and a consequent increase in AMPAR-mediated calcium influx, which leads to delayed neuronal death. This mechanism also appears to be involved in the response to a traumatic brain injury in vivo [44]. In the study by Bell et al. [44], the duration of the switch to GluR2-lacking AMPARs after mechanical insult was not directly investigated, but it is likely that a prolonged switch, similar to that observed after OGD [42], would be required to bring about changes in calcium influx that lead to neuronal death.

What determines the duration of the switch in subunit composition is still unknown. Probably the extremely high levels of glutamate released and therefore NMDAR activation in response to an OGD insult compared with LTP are responsible for the prolonged synaptic expression of GluR2-lacking AMPARs. Perhaps this leads to a change in PICK1 function, resulting in extended retention of GluR2 in recycling endosomes. The signalling pathways that modulate this switching mechanism will be a crucial topic for future research.

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