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# FUNCTIONAL CHARACTERIZATION OF A NOVEL CLATHRIN-INDEPENDENT ENDOCYTOSIS IN ASTROCYTES

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By

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# ABSTRACT

Endocytosis plays a fundamental role in regulating cell signaling, protein and lipid trafficking, and uptake of nutrients and pathogens in all eukaryotic cells. In astrocytes, endocytosis is particularly important for the uptake of nutrients. However, the molecular mechanisms underlying astroglial endocytosis are poorly understood. Using live fluorescence imaging with FM dye, we identify a rapid and constitutive endocytosis mechanism in cultured astrocytes. The endocytic uptake of FM dye is much faster than that of transferrin and the internalized FM dye is not colocalized with clathrin, indicating a clathrin-independent endocytic pathway. Moreover, this rapid endocytosis is caveolae-independent as indicated by intact FM dye uptake after blocking caveolae formation and lack of colocalization of FM puncta and caveolin.

In addition, rapid endocytosis is independent of dynamin but potently regulated by the early endosomal protein Rab5. After being internalized, FM dye is quickly sorted through early endosomes and transported to lysosomes. Interestingly, this rapid form of endocytosis is substantially regulated by intracellular $Ca^{2+}$. The neural and gliotransmitters ATP and glutamate induce an increase of

intracellular $Ca^{2+}$ in astrocytes, which greatly enhances rapid endocytosis. Furthermore, ATP is internalized through the same endocytic pathway as FM dye. This suggests a potential feedback control of the ATP signaling pathway in astrocytes. Moreover, amyloid $\beta$ peptide also significantly increases the rapid endocytosis through increasing intracellular $Ca^{2+}$ in astrocytes. These results suggest that astroglial cells posess a unique endocytic pathway that is independent of clathrin and dynamin but tightly regulated by intracellular $Ca^{2+}$ in response to physiological and pathological stimuli.

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# ABBREVIATIONS

| | |
|---|---|
| ARF | ADP-ribosylation factor |
| AMPA | $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| CNS | central nervous system |
| EGFP | enhanced green fluorescence protein |
| ER | endoplasmic reticulum |
| GABA | aminobutyric acid |
| GABAAR | GABA type-A receptor |
| GPI | glycosylphosphatidyl inositol |
| IGF | Insulin-like growth factor |
| NMDA | N-methyl-D-aspartic acid |
| PKC | protein kinase C |
| SNARE | soluble NSF attachment protein receptor |
| siRNA | small interfering RNA |
| TNF | tumor necrosis factor |

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# CHAPTER1 INTRODUCTION

## 1.1. General cell biology and classification of endocytosis

Endocytosis plays an important role in maintaining cellular activities by the uptake of bioactive molecules and recycling of proteins and lipids. In mammalian cells, different endocytic pathways are used in the uptake of different types of molecules. Generally, endocytosis can be classified as phagocytosis and pinocytosis. Phagocytosis, also called cell eating, refers to the internalization of large size of substance (>0.5 μm diameter). In mammalian cells, phagocytosis usually occurs in certain types of specialized cells, including monocytes and macrophages. It is considered a specific form of endocytosis that involves the internalization of solid particles, such as bacteria. Pinocytosis, on the other hand, refers to endocytic events of a smaller scale (<0.2 μm diameter) and it is widely used by cells to internalize membrane proteins, lipids, and fluid with soluble molecules. Pinocytosis can be further classified as macropinocytosis, clathrin-mediated and clathrin-independent endocytosis based on the size of the endocytic vesicles and the mechanism of vesicle formation (Conner and Schmid, 2003). Among pinocytosis, clathrin-mediated endocytosis is a well known endocytic event and has been extensively studied for decades (Mellman and Warren, 2000).

## 1.2. Endocytic pathway and molecular sorting

### 1.2.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is considered the most common endocytic pathway used by cells to uptake nutrients, signaling molecules, and receptors on the cell surface. Indeed, most forms of receptor-mediated endocytosis such as transferrin receptors, growth factor receptors, and G protein coupled receptors are clathrin-dependent (Sorkin and Von Zastrow, 2002). In all these cases, clathrin-mediated endocytosis is characterized by the formation of clathrin-coated pits of plasma membrane, which will further pinch off and produce clathrin-coated vesicles. After being internalized, the clathrin coat is quickly disassembled and vesicles are transported to a series of intracellular organelles depending on the cargo they are carrying (Mellman, 1996).

In clathrin-mediated endocytosis, usually the internalized vesicles first fuse with early endosomes and deliver their cargo to this major sorting station. Early endosomes have a slightly acidic pH (5.9-6), which may help certain types of receptors dissociate from their ligands. The receptors then are recycled to the cell membrane for further reuse through recycling endosomes budding from the early endosome. Cargos destined for degradation are sorted to late endosomes and lysosomes (Mellman and Warren, 2000). Interestingly, a recent study indicated that early endosomes include two distinct populations: an active and dynamic group that rapidly transforms into late endosomes, and another stable group that matures much more slowly. By using live-cell imaging, it was found that cargos destined for recycling and degradation are transported to these two distinct populations of early endosomes, respectively (Lakadamyali et al., 2006).

### 1.2.2 Clathrin-mediated synaptic vesicle recycling

In nerve terminals, neurotransmitter is packed in synaptic vesicles. It is released when synaptic vesicles fuse with cell membrane during $Ca^{2+}$ dependent exocytosis.

It has long been proposed that during synaptic vesicle turnover, some vesicle fusion involves the opening of a small fusion pore that opens and closes rapidly without full dilation. The existence of such “kiss and run” mode has been shown by a series of experiments using imaging and electrophysiological techniques (Gaiarsa et al., 2001; Aravanis et al., 2003). However, kiss-and-run is still not considered to be the major form of vesicle fusion at synapses, as it is usually observed under strong stimulation and only in certain types of neurons, such as the calyx of Held synapse (He and Wu, 2007).

In hippocampus, the majority form of vesicle fusion is still the “full collapse fusion”, during which the synaptic vesicles fully merge with and flatten into the presynaptic membrane (Granseth et al., 2006). In this classic fusion model, the retrieval of exocytosed vesicles is usually regulated by clathrin-mediated endocytosis. Indeed, shortly after exocytosis, clathrin-coated pits are assembled in the endocytic zone, which is dependent on extracellular $Ca^{2+}$ (Gad et al., 1998). The coated pits further bud toward the cytoplasm and form invaginations. Dynamin 1 is recruited around the narrow neck of the coated vesicles and further helps them dissociate from the cell membrane by constricting the neck. Another protein, endophilin, also plays an essential role in the early stage of clathrin-mediated endocytosis in never terminals. Microinjection of anti-endophilin antibodies disrupted recycling of synaptic vesicles by blocking the formation of clathrin-coated pits (Ringstad et al., 1999). In C. elegans, endophilin mutants are defective for synaptic vesicle recycling

(Schuske et al., 2003). More importantly, endophilin binds with voltage-gated $Ca^{2+}$ channels, and regulates the synaptic vesicle endocytosis in hippocampal neurons (Chen et al., 2003b). Thus, by interacting with $Ca^{2+}$ channels, the exocytic and endocytic machineries are coupled with each other in nerve terminals.

Like that in non-neuronal cells, the clathrin coats dissociate from synaptic vesicles after leaving plasma membrane in the endocytic zone. Some vesicles will be transported to early endosomes. There is also evidence showing that some uncoated endocytic vesicles may return directly to the release site to be ready for the next round of release (Brodin et al., 2000).

### 1.2.3 Caveolae-mediated endocytosis

Formation of clathrin-coated vesicles is the best-studied endocytic pathway and mainly responsible for recruiting cell-surface receptors in mammalian cells (Mousavi et al., 2004). However, non-clathrin endocytic pathways which do not require clathrin coat complexes for cargo transportation and budding are less characterized but equally important (Nichols and Lippincott-Schwartz, 2001).

The most well examined non-clathrin-mediated endocytic entry is the caveolae–dependent endocytic pathway. First described by Palade and Yamada in the early 1950s under electronic microscopy, caveolae are 50-100 nm, nonclathrin-coated, omega-shaped invaginations on the surface of the plasma membrane (Palade, 1955; Yamada, 1955). They can also exist as completely enclosed vesicles and as aggregates of several vesicles, which led to the idea that these structures are closely related to the endocytosis of macromolecules. In contrast to clathrin-coated pits, where a clathrin lattice is attached to the cytosolic tails

of membrane via adaptor proteins, the cytosolic side of caveolae is coated by proteins that directly bind to the lipid phase of the membrane.

This principal structural protein of caveolae is known as caveolin, a 21-24 kDa integral membrane protein which is essential for the caveolae formation (Rothberg et al., 1992). Multiple forms of caveolin including caveolin1, caveolin2 and caveolin3 have been identified and cloned. Although they share about 60% similarities in structure, they are quite different in specific properties and tissue distribution (Parton and Simons, 2007). Among them, the expression of caveolin-1 is highly correlated with the formation of invaginated caveolae. That is why caveolin-1 is used as the marker protein to identify the presence of caveolae exclusively. In addition, fluorescent conjugated caveolin1 was produced and widely used in live cell imaging to label the caveolae-mediated endocytic vesicles (Anderson, 1998; Pelkmans et al., 2004; Glebov et al., 2006). Interestingly, caveolin was found to not only provide the structural scaffolding for caveolae, but also play an important role in regulating the trafficking of those components related to cell-signaling events. Studies have revealed that caveolin binds directly with cholesterol, glycosphingolipids, and some lipid-modified signaling molecules (Pelkmans and Helenius, 2002; Parton and Simons, 2007).

A typical caveolae-mediated entry is the uptake of the simian virus 40 (SV40) during infection. It was found that SV40 enters into the stationary caveolae which are present on the cell membrane and the virus will be further internalized within a few minutes. After being endocytosed, SV40 is transported to caveolin-1 enriched cellular compartments called caveosomes and then accumulates in the ER (Pelkmans et al., 2001; Pelkmans and Helenius, 2002; Pelkmans et al., 2002). However, recent studies also indicated the presence of a nonclathrin and noncaveolae

entry for SV40 (Damm et al., 2005). Other than SV 40, caveolae were also found to be involved in the uptake of echovirus 1, cholera-toxin-binding subunit (CTxB) and human histamine $H_1$-receptor (Orlandi and Fishman, 1998; Self et al., 2005; Parton and Simons, 2007).

Caveolae are abundant in almost all kinds of tissues. Cameron *et al.* first found that in cultured type I astrocytes there are morphologically identifiable caveolae, which are coated predominantly by caveolin-1 (Cameron et al., 1997). Previous studies indicated that caveolin may play a role in cholesterol transportation. For example, caveolin was found to be involved in the rapid trafficking of cholesterol from the ER to the cell surface as newly synthesized cholesterol was delivered to the plasma membrane in a caveolin-dependent manner. The newly delivered cholesterol then rapidly flowed from caveolae to non-caveolae membrane (Smart et al., 1996). Interestingly cholesterol was identified as a synapse-promoting factor secreted by astrocytes (Mauch et al., 2001) and it was proposed that instead of synthesizing cholesterol by themselves, neurons mainly import cholesterol from astrocytes (Pfrieger, 2003). Thus, caveolae in astrocytes may play a role in regulating the transportation of cholesterol which is critical to synapse formation.

### 1.2.4 Clathrin- and caveolae-independent endocytosis

Besides clathrin and caveolae-dependent endocytic entries, it is widely accepted that there are other endocytic pathways that don't rely on the formation of clathrin coated pits or caveolae. Interestingly, some cargos were shown to be endocytosed via nonclathrin and noncaveolae entries. For example, it was shown that cholera toxin B can be endocytosed at least partially via a nonclathrin and noncaveolae

mechanism (Simpson et al., 1998; Kenworthy et al., 2000; Glebov et al., 2006).

Another example of nonclathrin and noncaveolae endocytic event is the internalization of glycosylphosphatidyl inositol (GPI)-anchored proteins. GPI is a glycolipid that can bind to the C-terminus of certain proteins during post-translational modification by the action of GPI transamidases. GPI-anchored proteins have very diverse functions. In mammalian cells GPI-anchored proteins play an important role in development because several cell adhesion molecules, such as cadherin, are GPI-anchored (Kawagoe et al., 1996). Recently it was shown that GPI anchored proteins are endocytosed to the recycling endosomes in a clathrin-, and caveolae-independent manner but regulated by cdc42 (Sabharanjak et al., 2002).

Molecules that are essential to nonclathrin and noncaveolae dependent endocytic pathways are currently under investigation. A recent study found that flotillin 1 plays an important role in such processes. Flotillin 1 is associated with lipid rafts forming multimeric scaffolds on the cytoplasmic membrane (Solis et al., 2007). In addition, flotillin-1 enriched regions on the plasma membrane, which are distinct from clathrin-coated pits and caveolae, seem to be endocytosed into cells. Moreover, downregulation of flotillin 1 by siRNA disrupted the nonclathrin and noncaveolae dependent endocytosis of GPI anchored proteins (Glebov et al., 2006). These results indicate that flotillin 1 could be a major regulator and even may serve as a structural molecule in endocytic entry that is both clathrin and caveolae independent.

In addition to flotilin-1, ARF1, a member of ARF (ADP-ribosylation factor) family was also found to actively regulate the nonclathrin and noncaveolae endocytic pathway (Kumari and Mayor, 2008). The

ADP-ribosylation factor (ARF) family of proteins is part of the Ras superfamily of small GTPases. Previously ARF1 was reported to be involved in the regulation of secretory membrane transport. Indeed, the retrograde transportation, from the Golgi to the endoplasmic reticulum (ER) is mediated by ARF1 which help recruit the COPI to budding the transport vesicles (Vasudevan et al., 1998; Bonifacino and Glick, 2004). Current studies show that ARF1 is present on the plasma membrane and play an essential role in regulating the endocytosis of nonclathrin and noncaveolae mediated GPI anchored proteins. Depleting ARF1 by overexpressing a dominant negative form of ARF1 or treatment with ARF1 siRNA largely blocked entry of GPI anchored proteins. It is proposed that when ARF1 is activated, it binds with its downstream effector ARHGAP10 resulting in the release of Cdc42 into the cytosol. The activated cdc42 can further facilitate the internalization of GPI anchored proteins (Kumari and Mayor, 2008).

Overall, although it is quite clear that besides the clathrin and caveolae-mediated endocytosis, there is endocytic uptake existing in mammalian cells that does not rely on the formation of either clathrin coated pits or caveolae. However, certain molecules, like ARF1 or Cdc42, which belongs to small GTPase superfamily may work with other membrane proteins, such as flotillin 1, to regulate the activity of actin and initiate the vesicle endocytosis without clathrin pit and caveolae.

### 1.2.5 Role of dynamin in endocytosis

Dynamin is a 100kDa GTPase which is essential to vesicle formation in clathrin mediated endocytosis (Hinshaw, 2000). It was found that when cells were transfected with dynamin mutants deficient in GTP binding (K44A), the internalization of transferrin or EGF receptors which is clathrin

mediated was largely blocked. In addition, coated pits were found not to be able to completely bud off from the cell surface suggesting that dynamin mutants block the endocytosis in its very early stage (Herskovits et al., 1993; van der Bliek et al., 1993; Damke et al., 1994). Indeed, immunogold labeling of dynamin under electron microscopy shows that dynamin is located on the clathrin lattice in the GDP binding status but redistributes to the necks of the clathrin-coated pits upon GTP binding (Muhlberg et al., 1997; Warnock et al., 1997). Based on these finds, it is believed that during clathrin mediated endocytosis, dynamin-GTP is recruited at the neck of invagination. Upon GTP hydrolysis it further restricts the neck and help vesicles dissociate from the cell surface (Hinshaw, 2000).

In addition to clathrin mediated endocytosis, dynamin is also found to regulate some clathrin-independent endocytic events. For example, cells transfected with the mutant dynamin show defects in caveolae internalization (Oh et al., 1998). In addition, the overexpression of mutant dynamin also largely disrupted the uptake of cholera toxin B, which is known to be endocytosed by caveolae regulated endocytic pathway. Indeed, very much like the location of dynamin present in clathrin coated pits, under the electron microscopy, dynamin was also found to be enriched in the necks of caveolae invaginations (Oh et al., 1998).

### 1.2.6 Role of Rab5 in endocytosis

Besides dynamin, other molecules like ras-like small GTP-binding proteins, also play roles in the initiation and regulation of endocytosis. For example, Rab5 has been shown to interact with early endosomes and plasma membrane (Zerial and McBride, 2001). In addition, Rab5 plays a critical role in early endosomal biogenesis regulating the fusion of

endocytic vesicles to early endosomes and the homotypic fusion of endosomes. EEA1 is the Rab5 effector that is required for tethering/docking of early endosomes (Christoforidis et al., 1999). Overexpression of the constitutively active mutant of Rab5 which enhances homotypic endosome fusion results in dramatic enlargement of early endosomes. In contrast, cells overexpressing the inactive mutant form of Rab5 failed to maintain normal receptor-mediated endocytosis, and the formation of early endosomes was largely disrupted (Stenmark et al., 1994; Ceresa et al., 2001)..

More importantly, Rab5 is also shown to be essential to the formation of internalized vesicles. It was proposed that when clathrin is recruited to the vesicle membrane, GDI helps deliver Rab5 to the membrane to promote the membrane fission (Bucci et al., 1992; McLauchlan et al., 1998). This may also explain why the overexpression of dominant negative Rab5 can disrupt the clathrin-mediated endocytosis. However, the detailed mechanism of how Rab5 regulates the initiation of endocytosis is still not clear yet.

Serving as membrane organizers, different members of the Rab family show distinct subcellular locations and thus are responsible for different parts of intracellular transportation and trafficking (Zerial and McBride, 2001). For example, as mentioned before, Rab5 is largely distributed on plasma membrane and early endosomes, and it regulates the fusion of endocytic vesicles to early endosomes. Another member of the Rab family, Rab7, however, is primarily associated with late endosomes, regulating the maturation of early endosomes to late endosomes (Zerial and McBride, 2001)

## 1.3 Endocytosis in astrocytes

### 1.3.1 Optical detection of vesicle endocytosis by FM dye

FM dye is amphipathic molecules with a charged pyridinium group on one end and a lipophilic dialkylaminophenyl group at the other end (Cochilla et al., 1999). This water-soluble dye, which is nontoxic to cells and virtually nonfluorescent in aqueous medium, is believed to insert into the outer leaflet of the cell membrane and then becomes intensely fluorescent. At the meantime the nonspecific staining of cell-surface membranes can be largely washed off before imaging. In neuronal preparations, during stimulation such as high concentration of extracellular potassium, exocytosis causes vesicular membrane to fuse with plasma membrane, and FM dye binds to plasma membrane. Following exocytosis, FM dye is trapped inside synaptic vesicles through endocytosis. After washing out excess dye from the extracelluar buffer, endocytosed vesicles can be viewed under fluorescent microscopy. FM dye is widely used to study endocytosis, vesicle trafficking, and organelle organization in living animal cells, especially in neuronal preparations to study synaptic transmission, synaptic plasticity, and synaptogenesis (Betz et al., 1992; Ahmari et al., 2000; Friedman et al., 2000; Ahmari and Smith, 2002; Chen et al., 2003b). After internalization, the dye is distributed to different organelle membranes, probably via the vesicle trafficking network and thus components of secretory pathways become labeled.

In addition to being considered as a powerful and convenient tool to investigate the vesicle trafficking and synaptic transmission in neuronal studies, FM dyes have also been widely used to study endocytosis, vesicle trafficking and organelle organization in other kind of cells,

including cultured astrocytes (Bolte et al., 2004; Zhang et al., 2007).

### 1.3.2 Endocytosis in astrocytes.

Endocytosis has numerous important functions in mammalian cells: it is critical for controlling the protein–lipid composition of the plasma membrane and the uptake of nutrients as well as pathogens, and it also plays an important role in the regulation of cell signaling (Cavalli et al., 2001). However, the biological significance of endocytosis in astrocytes has not been thoroughly investigated.

It was first reported that following an intravenous injection of horseradish peroxidase, reaction product rapidly appeared in glial cell cytoplasm, and numerous membrane-bound vacuoles of various size and morphology were identified under the electron microscope. This suggested that endocytosis normally occurs in astrocytes (Theodosis, 1979). Receptor-mediated endocytosis such as the internalization of Insulin-like growth factor (IGF) in astrocytes was then identified (Auletta et al., 1992). Recent studies found that cultured astrocytes have both caveolae and clathrin-coated vesicles widely distributed throughout the cytoplasm (Megias et al., 2000). In addition, in cultured rat astrocytes after being incubated with cationized ferritin, a high molecular weight endocytic tracer, superficial labeling was already be observed in some vesicular compartments near the cell membrane. This indicates the presence of rapid endocytosis in the astrocytes (Silva et al., 2001). Most recently it was reported that after incubating with FM dyes for hours, cultured astrocytes can uptake FM dyes which gradually accumulated in the lysosomes (Zhang et al., 2007).

Although the studies summarized above have clearly demonstrated the presence of endocytosis in astrocytes, its molecular mechanism and function are not clear yet. For example, how many endocytic pathways are there in astrocytes? Can endocytosis in astrocytes take up neurotransmitters? How is glial endocytosis regulated?

## 1.4 Intracellular $Ca^{2+}$ and neuron-glia interaction

### 1.4.1 Overview of astrocyte functions

In traditional views, astrocytes are considered as passive partners to neurons in the brain mainly because they are not electrically excitable. They function in regulating extracellular glutamate, via glutamate transporters and recycling glutamate via glutamine and intermediates of the tricarboxylic acid cycle. By clearing excess glutamate from the extracellular space, astrocytes protect against excitotoxic glutamate that can cause neuronal cell death (Auld and Robitaille, 2003).

One well known function of glial cells is that they play an important role in the formation and maintenance of blood-brain-barrier (BBB) that functions in the homeostatic regulation of the brain microenvironment critical for the healthy function of the CNS (Prat et al., 2001). It was found that anatomically the endfeet of astrocytes form a lacework of endothelium. In addition to this, astrocytes are connected to each other by gap junctions that provide electronic and ionic continuity among the spatially extended astrocytes. Moreover, astrocytes are also found to wrap around many of neuronal cell bodies as well as dendrites while their fine processes closely associated with individual synaptic terminals. Indeed, in the rat hippocampus, 57% of the axon-spine interfaces are tightly associated with

surrounded astrocytes (Ventura and Harris, 1999). Because the same astrocyte can make contact with neurons and capillaries, presumably it has the potential to transport nutrients and metabolites between the blood supply and neurons that it connects (Araque et al., 1999).

### 1.4.2 Astrocytes promote synaptogenesis

Synaptogenesis was once considered a purely neuronal process. However, there is accumulating evidence suggesting that glia-derived molecules, soluble or membrane-bound, are actively involved in synapse formation. In 1997, Pfrieger and Barres first investigated whether glial cells could enhance synaptic efficacy of rat retinal ganglion cells (RGCs) *in vitro*. They found that with the support from glial cells which grow underneath, the level of spontaneous excitatory synaptic activity was greatly enhanced. In addition, these effects were at least partially caused by one or more soluble factors released by astrocytes and oligodendrocytes (Pfrieger and Barres, 1997). Later on, it was reported that astrocytes can directly promote the synapse formation by increasing the number of functional synapses. At the meantime astrocytes remarkably increase the number of puncta containing both pre and postsynaptic proteins (Ullian et al., 2001). In addition to this, by using FM dye to label active terminals and electrophysiological recordings of autaptic currents, Mauch et al. showed that glial factors also enhance the efficacy of presynaptic transmitter release: Treating neuronal cultures with glia-conditioned medium (GCM) accelerated the stimulation-induced loss of FM fluorescence from individual presynaptic terminal and also greatly enhanced the frequency of autaptic events (Mauch et al., 2001). All these findings strongly indicated that soluble glia-derived factors promote synapse development.

Since then, efforts had been put into the identification and isolation of astrocyte-derived factors that can promote synapse formation. For example, it has been found that astrocytes constitutively secrete TNF$\alpha$, tumor necrosis factor, which increase the surface expression of AMPA receptors and promote the synaptic transmission (Beattie et al., 2002). Pfrieger's group first reported that cholesterol could be one of the glia-derived factors that have synaptogenesis promoting effects. They found that supply of cholesterol to RGCs increases the frequency of spontaneous EPSCs to a similar level as glia conditioned medium. In addition, cholesterol treatment alone also increased the number of synapses in a similar pattern as it is with the treatment of glia conditioned medium. More convincingly, reduction of the cholesterol concentration in glia conditioned medium by the cholesterol synthesis inhibitor strongly reduced its synaptogenic effect (Mauch et al., 2001; Nagler et al., 2001). The effect of cholesterol was further examined and confirmed by a series of independent experiments. It was further identified that cholesterol directly promotes the differentiation which is essential for continuous synaptogenesis (Goritz et al., 2002; Goritz et al., 2005). These data suggest during development, after differentiation of astrocytes, neurons tend to reduce their cholesterol synthesis and rely mostly on cholesterol synthesized and transported by astrocytes (Pfrieger, 2003). In addition to cholesterol, other molecules like estrogen were also found to have similar effects as cholesterol, which needs to be confirmed by future studies (Hu et al., 2007). Furthermore, recent studies by Barres's group identified that thrombospondins-1 and thrombospondins-2 expressed by astrocytes promote CNS synaptogenesis both in vitro and in vivo. Interestingly, thrombospondins are expressed in the postnatal brain and promote synapse formation in immature neurons. (Christopherson et al., 2005)

However, the identification of this factor does not preclude the contribution of membrane-bound factors or the direct contact between neurons and astrocytes in the enhancement of synaptic efficacy. Previous studies by Song *et al*. reported that primary hippocampal astrocytes alone are sufficient to promote neurogenesis from adult stem cells and both diffusible and membrane-bound factors are taking effect (Song et al., 2002b). Most interestingly it was shown that indeed when growing together, a local astrocytes-neuron contact via integrin can facilitate excitatory synaptogenesis throughout the neuron. In addition, these effects are mediated by the activation of PKC (Hama et al., 2004).

### 1.4.3 Neurons form synapses with glial cells

It has been identified that in brain slices, stimulation of excitatory axons in the hippocampus triggered inward currents in oligodendrocyte precursor cells (OPCs) indicating that neurons can directly form synapses with certain types of glias. More convincingly, OPCs were found being apposed to presynaptic neurotransmitter release sites and forming synaptic clefts (Bergles et al., 2000). Moreover, recent research further found that these neuron-glia synapses show activity-dependent plasticity which is similar to long-term potentiation (LTP) at excitatory synapses (Ge et al., 2006). These results suggest that by forming synapses, neurons and glias may conduct direct interaction with each other and this rapid neuron-glia signaling may allow rapid feedback regulation of neuronal functions.

### 1.4.4 Intracellular $Ca^{2+}$ oscillation in astrocytes

Changes in the intracellular $Ca^{2+}$ level are considered to be a critical intracellular signal mediating a variety of biological activities in cells. Studies in cell culture first showed that local application of glutamate to cultured astrocytes triggered a $Ca^{2+}$ elevation that propagated as a wave through adjacent astroglia network (Cornell-Bell et al., 1990; Glaum et al., 1990; Brune and Deitmer, 1995). More interestingly, in brain slices, electrical stimulation in neurons which release glutamate results in an increase of intracellular $Ca^{2+}$. This indicates that *in situ*, astrocytes are able to respond to neurotransmitter release, such as glutamate, from nerve terminals with an increases in intracellular $Ca^{2+}$ (Dani et al., 1992). In addition, the increase of intracellular $Ca^{2+}$ is due to glutamate binding to metabotropic glutamate receptors (mGluRs) on astrocytes (Porter and McCarthy, 1996; Pasti et al., 1997).

### 1.4.5 Astrocytes release glutamate

Exocytosis is defined as the fusion of an intracellular trafficking vesicle with the plasma membrane. Every eukaryotic cell is dependent on exocytosis for growth and differentiation because it is considered to be the only mechanism by which a cell can add additional membrane to the cell surface (Burgess and Kelly, 1987). Recent data suggest that in astrocytes exocytosis may be responsible for releasing glutamate and possibly other molecules that can actively regulate neuronal activity.

It is known that astrocytes can modulate synaptic transmission by releasing chemical messengers such as ATP and glutamate. For example,

Haydon and colleagues showed that activation with bradykinin increases $Ca^{2+}$ in surrounding neurons in an NMDA receptor-dependent manner, implying that glutamate could be released by activated astrocytes. Moreover, it was shown that the release of glutamate from astrocytes was coupled with the oscillation of intracellular $Ca^{2+}$ (Pasti et al., 2001).

However, the detailed mechanisms underlying the release of these transmitters from astrocytes are not well-defined. Araque *et al.* showed that $Ca^{2+}$-dependent glutamate release from astrocytes is a SNARE protein-dependent process, suggesting that astrocytes store glutamate in vesicles which are released through an exocytic pathway (Araque et al., 2000). The direct evidence supporting this idea was presented by Haydon's group. It was shown that in astrocytes, the activation of metabotropic glutamate receptors evoked a calcium elevation and caused an increase in the cell surface area which may indicate the simultaneous fusion of vesicles with the plasma membrane. More convincingly when the formation of SNARE complexes was disrupted, the release of glutamate from astrocytes was largely blocked (Zhang et al., 2004).

Recent studies, however, suggested that the glutamate can be released in a "kiss-and-run" pattern, a distinct exocytic model in which exocytic vesicles form a fusion pore with the plasma membrane that allow transmitter release instead of fully fusing with the plasma membrane and collapsing (Chen et al., 2006).

### 1.4.6 Astrocytes release ATP

Besides glutamate, astrocytes also release other transmitters, such as ATP. It has been first reported by Kater's group that ATP is released during $Ca^{2+}$ propagation by astrocytes as an extracellular

message (Guthrie et al., 1999). Later on, it was found that in hippocampal cultures, astrocytes may release ATP, which suppresses glutamatergic synapses via P2Y receptors located on presynaptic terminals. In addition, the ATP metabolite adenosine also contributes to the process but by interacting with A1 receptors in neurons (Zhang et al., 2003).

Similar to the release of glutamate, it is believed that the release of ATP is via a SNARE-dependent exocytic pathway, because ATP release from astrocytes is sensitive to tetanus toxin and is blocked by expression of a cytosolic SNARE domain (Pascual et al., 2005). Most recently it was reported that ATP is continuously endocytosed and accumulated in lysosomes in cultured astrocytes. Upon stimulation, the ATP restored in the lysosomes can be released directly from lysosomes via exocytosis (Zhang et al., 2007). Indeed, previous research has shown that conventional lysosomes can fuse with the plasma membrane in response to a change in intracellular $Ca^{2+}$. Although the biological significance of such lysosome exocytosis is not clear yet, it is thought to be a way that cells use to provide extra membrane for repairing plasma membrane wound (Luzio et al., 2007). New data provided by Zhang et al, however, suggest that the lysosome exocytosis releasing ATP may play an important role in astrocyte-neuron interaction. In short, neurotransmitter released by exocytosis in astrocytes, can actively regulate neuronal activity and thus could be one of the important mechanisms mediating the glia-neuron interaction.

### 1.4.7 Astrocytes regulate neuronal activities

As discussed above, the elevation of intraglial $Ca^{2+}$ may further lead to the glutamate release from astrocytes, which can affect the adjacent neurons (Parpura et al., 1994). Similar results were confirmed in

brain slices by Pasti et al (Pasti et al., 1997). More importantly, it was demonstrated later that the activation of astrocytic mGluRs will trigger the increase of intracellular $Ca^{2+}$. And the oscillation in astrocytes caused a delayed neuronal $Ca^{2+}$ signal mediated by ionotropic GluRs (Pasti et al., 1997). This is the first direct evidence suggesting that astrocytes not only receive biological signals from neurons but also actively regulate the neuronal activities upon stimulation.

Indeed, it has been shown that $Ca^{2+}$ dependent glutamate release from astrocytes increased the frequency of synaptic currents by activating presynaptic NMDA receptors (Araque et al., 1998a; Araque et al., 1998b). Neurotransmitter released from astrocytes is also proven to regulate synaptic activities in more intact preparations. For example, in brain slices, glutamate released by astrocytes may bind to presynaptic neuronal mGluRs and thus increase the frequency of spontaneous EPSCs in CA1 pyramidal neurons (Fiacco and McCarthy, 2004).

Beside glutamate, ATP released by astrocytes also plays an essential role in regulating neuronal activities. For example, in hippocampal cultures, ATP released by astrocytes may depress glutamatergic neuronal transmission, which is caused by the ATP acting on the presynaptic P2Y receptors (Koizumi et al., 2003). Moreover, in brain slices, it was shown that, ATP released by astrocytes binds to P2Y receptors on interneurons and further strengthen the synaptic inhibition (Bowser and Khakh, 2004)

Based on the preceding discussion, it is quite clear astrocytes can release different kinds of neurotransmitter to communicate with the neuronal network and regulate neuronal transmission. Glutamate released from astrocytes can potently enhance excitatory synaptic activities. On the contrary, when ATP is released, it will facilitate the inhibition of neuronal

transmission. In addition, the change of intracellular $Ca^{2+}$ in astrocytes plays a unique role in initiating the transmitter release and coordinating the interaction between astrocytes and neurons.

Overall, there is more and more evidence showing that astrocytes are active partners of neurons. Indeed astrocytes are capable of not only receiving neuronal message but also modulating synaptic transmission in sophisticated ways. Based on the complexity of astrocyte-neuron interaction, studies have demonstrated that during the development of neurodegenerative diseases, astrocytes are severely affected and the malfunction of astrocytes may lead to further neuropathologic changes in the progress of these diseases.

## 1.5 The role of astrocytes in Alzheimer's disease

Alzheimer's disease is a common neurodegenerative disorder characterized by progressive neuronal cell death, synaptic disassembles, and neuronal network dysfunction. One of its major pathological changes involves the constitutive accumulation of extracellular senile plaques which are composed largely by Aβ amyloid peptide. In addition, during the development of Alzheimer's disease, the accumulation of Aβ amyloid peptide has been shown to be one of the major neurotoxic elements that is directly responsible for the neuronal loss and dysfunction (Morgan et al., 2004).

### 1.5.1 The production of Aβ peptide

The human Aβ-amyloid gene is located on chromosome 21 and encodes a transmembrane protein called amyloid precursor protein (APP).

APP is a membrane-spanning glycoporotein expressed in the brain and it can be processed by different proteolytic pathways resulting in different forms of products. In the non-amyloidogenic pathway, the α-secretases cut APP to produce an extracellular and soluble ectodomain of APP (sAPPα). Since the cleaving site by the α-secretases is located in the middle of the Aβ region, it prevents the formation of Aβ. When APP is cleaved sequentially by β- and γ-secretase complexes, however, it will generate Aβ as the amyloidogenic element (Morgan et al., 2004). The cleavage by γ-secretases produces a full length Aβ peptide. Not surprisingly, mutations in APP can lead to pathogenesis of Alzheimer disease, especially for early-onset Alzheimer disease (Tanzi et al., 1996; Selkoe, 2001; Goedert and Spillantini, 2006). Most Aβ is 40 aa in length (Aβ1-40) but a small number of them is 42 aa in length (Aβ1-42). Although Aβ1-42 may only account for 10% of total intact Aβ peptides, it is believed to be more amyloidogenic and predominantly deposited in the cerebral plaques during development of Alzheimer disease (Caselli et al., 2006).

### 1.5.2 Aβ peptide and its neuronal toxicity

Aβ peptides were shown to be directly related to the neuronal degeneration of rat hippocampal and cortical neurons *in vitro* and *in vivo*. Treatment of cultured neurons with 25 -100 μM Aβ in a few days can induce significant neuronal apoptosis, featured with neurite beading, condensed chromatin, and DNA fragmentation (Yankner et al., 1990;

Loo et al., 1993; Watt et al., 1994). However, the mechanism by which Aβ exert its toxic effects is still not clear yet. It has been proposed that neuronal toxicity to Aβ occurs via different mechanisms: Aβs can promote free radical formation inducing neuronal damage (Behl et al., 1994), or alter calcium levels, thereby promoting susceptibility to excitotoxic damage (Mattson et al., 1992). In some cases, Aβ was found to downregulate anti-apoptotic protein, bcl-2 and increase the expression of bax, a protein known as an apoptosis promoter (Paradis et al., 1996). Besides that there is strong evidence showing that Aβs' toxicity is related to oxidative stress. For example, when being exposed to Aβ, the activities of various enzymes that are highly sensitive to oxidative modification were greatly decreased (Kish, 1997).

In addition to neurons, recent studies of the pathological changes in astrocytes have demonstrated that astrocytes, as close partners of neurons, play essential roles in regulating the neuronal toxicity of Aβ and the development of Alzheimer's disease.

### 1.5.3 Astrocytes regulate Aβ neuronal toxicity

Astrocytes play a variety of roles in the pathogenesis of Alzheimer's disease. When Aβ-treated hippocampal culture was grown with untreated healthy glia conditioned medium, it was found that the Aβ toxicity to neurons was greatly reduced causing less neuronal death. This indicated that glia, when not being exposed to Aβ, could protect surrounding neurons from the neuronal toxicity caused by Aβ. Since the mechanism of

how Aβ cause neuronal loss is still not clear, it is even harder to understand how glia is able to reduce Aβ's neuronal toxicity. However, it has been proposed that glia conditioned media may contain certain types of secretory factors, for example, BDNF and NGF, which are neurotrophic and may help maintain the integrity of the neuronal network (Takuma et al., 2004). Alternatively, by supplying antioxidants and/or antioxidant enzymes, astrocytes may protect neurons against the toxicity of oxidants generated by Aβ treatment (Dringen et al., 2000). Intriguingly, in hippocampal neuron-glia mixed culture, when both glias and neurons are exposed to the Aβ, the neuronal damage seemed to be enhanced. Thus astrocytes, in the absence of Aβ, are able to reduce the neuronal toxicity of Aβ possibly by helping remove the oxidants for affected neurons. However, in the hippocampal-glia mixed culture, Aβ treatment not only abolishes astrocytes' neuroprotective effect, but also enhances the neuronal loss (Malchiodi-Albedi et al., 2001; Domenici et al., 2002; Paradisi et al., 2004). These data indicate that Aβ has substantial impact on astrocytes and the Aβ-astrocyte interaction may play an essential role in regulating the toxicity of Aβ during the development of Alzheimer disease.

### 1.5.4 Aβ affects astroglial intracellular $Ca^{2+}$

The intracellular $Ca^{2+}$ is considered to be one of the most important signals in glia that can regulate a variety of biological reactions. More importantly, it also plays an essential role in glia-neuron interaction (Haydon, 2001). While Aβ treatment seemed to have no obvious effect on

the intracellular $Ca^{2+}$ in cultured neurons, application of either the full length peptide (Aβ1-42) or the 25–35 aa fragment (Aβ 25-35) can trigger a complex of intracellular $Ca^{2+}$ oscillation. In addition, in $Ca^{2+}$ free bath solution where no extracellular $Ca^{2+}$ is present, Aβ treatment failed to cause intracellular $Ca^{2+}$ oscillation. This indicated that the intracellular $Ca^{2+}$ response to Aβ in astrocytes is dependent on the extracellular $Ca^{2+}$ rather than the intracellular $Ca^{2+}$ store (Abramov et al., 2003).

Although Aβ treatment can cause intracellular $Ca^{2+}$ oscillation in astrocytes, the underlying mechanism is still not clear. One possible mechanism is that Aβ can interact with pre-existing $Ca^{2+}$ channels that are selectively expressed in astrocytes. Alternatively, Aβ itself can form channels on cell membrane. Interestingly, when incorporated and fusing with liposomes, Aβ formed channels that were permeable to $Ca^{2+}$ and other ions. In addition, the structural evidence for these channels in lipid bilayer membrane was revealed by atomic force microscopy. When Aβ fuses with the liposomes, its monomeric structure converted into a tetrameric channel-like structure (Lin et al., 2001). In addition, zinc can prevent the formation of Aβ channels and heavy metal chelator such as clioquinol has the similar effect. These results provide promising approaches which can lead to clinical application in patients with Alzheimer's disease (Cherny et al., 2001; Regland et al., 2001).

Although these data were favoRable to the Aβ channel forming theory, it is hard to explain how these channels can lead to oscillatory and transient $Ca^{2+}$ changes. If the channel was permanently inserted and stably present on the cell membrane, one should expect a monotonic

phase of intracellular $Ca^{2+}$ increase.

### 1.5.5 Aβ and oxidative stress in astrocytes

It has been widely accepted that the pathogenesis of the Alzheimer's disease involves oxidative stimulation which has damaging effect on certain proteins. Antioxidants, such as glutathione (GSH), and antioxidant enzymes are expressed at high concentrations in astrocytes, which may protect the surrounding neurons from oxidative toxicity (Dringen et al., 2000). Indeed, Aβ has been shown to increase the amount of reactive oxygen species (ROS) and the antioxidant mechanism is compromised by Aβ at the same time. For example, in glia, Aβ increases the rate of ROS generation which is dependent on $Ca^{2+}$. In addition, overnight exposure to Aβ leads to the depletion of GSH, which is one of the major antioxidants, in both astrocytes and neurons (Abramov et al., 2004). Since neuronal viability was shown to be improved by the supply of GSH precursors, it suggests that GSH depletion in astrocytes may impair their neuroprotection effect and lead to the progression towards cell death in both astrocytes and neurons (Abramov et al., 2004).

Overall current literatures have clearly pointed out that during Alzheimer's disease the toxicity of Aβ affects both neurons and astrocytes. When normal function of astrocytes is disrupted and the astrocyte-neuron interaction is disturbed, the pathologic changes of the disease could be extended.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1 Astroglial Culture

Astroglial cultures were prepared from new born Sprague-Dawley rats (P2-P3) similar to previously described (McCarthy and de Vellis, 1980; Jiang et al., 2004) In brief, cortex tissue was dissected out and incubated for 30 min in 0.05% Trypsin-EDTA solution. After enzyme treatment, tissue blocks were triturated gently and dissociated cells were plated onto 25 $cm^2$ flasks and maintained for 5-7 days. During the first week after plating, flasks were shaken rigorously to remove neurons and other non-astrocytic cells until astrocytes reached confluence before being seeded onto coverslips. The culture medium contained 500 ml MEM (GIBCO), 5% fetal bovine serum (Hyclone), 100 mg $NaHCO_3$, 20 mM D-glucose, 2.5 mM L-glutamine, and 25 unit/ml Penicillin/Streptomycin. Cells were maintained in 5% $CO_2$ incubator at 37 °C for 2-3 weeks. After being seed onto coverslips, cells are growing another 2-3 days till they rearch 80-90% confluent and Ara-C is add to stop the over proliferation.

## 2.2 Cell transfection

Cells were transfected with a modified $Ca^{2+}$-phosphate transfection protocol developed in our lab (Jiang et al., 2004; Jiang and Chen, 2006). DNA/$Ca^{2+}$-phosphate precipitate was prepared by using the Clontech CalPhos™ Mammalian Transfection Kit (BD Bioscience, Palo Alto, CA). Cells were incubated in the presence of the precipitate for 30-60 min in 5% $CO_2$ culture incubator. After incubation, the precipitate was removed by

washing coverslips with 10% $CO_2$ incubated transfection medium. Usually after 24-48 hrs of transfection, cells were taken out for experiments.

## 2.3 FM staining

Astrocytes were used after 2-20 days in vitro (DIV), with the majority between 7–14 DIV. Coverslips were transferred into a perfusion chamber mounted on a microscope stage and continuously perfused with a bath solution containing 128 mM NaCl, 30 mM D-glucose, 25 mM Hepes, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.3, adjusted with NaOH). For most experiments, cells were stained with bath solution containing 10-20 μM FM 1-43 or FM 4-64 for 1.5-5 min and then washed with ADVASEP-7 for 5 min to reduce the background signal (Kay et al., 1999). Fluorescence signal of FM dye was visualized on a Nikon TE-2000-S microscope and analyzed using Simple PCI imaging software (C-Imaging, Pittsburgh). To quantify the intensity of fluorescent signals, the mean intensity of each individual astrocyte was measured with Simple PCI imaging software and then averaged across different cells. The background signal in non-astrocytic area was subtracted. For easy comparison, the fluorescent intensity of testing groups were normalized to the control group. Student's *t* test was used to test statistical significance between control and experimental groups.

## 2.4 $Ca^{2+}$ imaging

Cells were incubated in bath solution (128 mM NaCl, 30 mM D-glucose, 25 mM Hepes, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.3, adjusted with NaOH) containing 10 μM fura-2 AM (Molecular Probes,

Eugene, OR) for 45 min at 37°C . After dye loading, coverslips were transferred to a perfusion chamber mounted on a Nikon TE-2000-S inverted microscope with a 40x objective and a high 340/380 nm transmittance filter for $Ca^{2+}$ ratio imaging (Chroma Technology Corp, VT). Cells were then washed for 15 min in bath solution with a perfusion rate of 1-2 ml/min. Images were collected from randomly selected areas in astrocytes. Calcium signals were presented as ratios of the fluorescent emission signals after excitation at 340 nm and 380 nm correspondingly, and processed by Simple PCI imaging software (C-Imaging, Pittsburgh).

## 2.5 Electron Microscopic Analysis

Coverslips containing cultured cortical astrocytes were rinsed twice in PBS and fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer containing 4% sucrose for 1 hr on ice, with a change of fresh fixative in between. After a brief rinse with PBS, the cells were post-fixed with 1% of reduced $OsO_4$ for 1 hr, en bloc stained in 1% aqueous solution of uranyl acetate for 30 min, and then dehydrated with a series of graded ethanol. The coverslips were placed upside down on embedding media and polymerized in Epon/Araldite at 70°C for 24 h. Resin-embedded cells were recovered by removing the glass coverslips and glued to blank resin blocks. Ultrathin sections were cut from the very surface of blocks and collected on 200-mesh, Formvar-coated copper grids (Electron Microscopy Sciences, Fort Washington, PA), and contrasted with uranyl acetate and lead citrate. Images were recorded with a JEOL 1200 XII transmission electron microscope at 80 kv and 20,000x.

## 2.6 Immunostaining

For immunostaining, cells were washed three times in PBS, fixed in 4% paraformaldehyde for 12 min, and permeabilized for 5-15 min with 0.01-0.2% triton in PBS containing 10% donkey serum. After a brief wash in PBS, cells were incubated with primary antibodies overnight at 4°C. The secondary antibodies conjugated with Alexa Fluorophores were used to detect primary antibodies. Fluorescence signals were visualized with an Olympus confocal microscope (Olympus Fluoview FV 1000) or a Zeiss Axioplan 2 microscope.

Primary antibodies: Rabbit anti-GFAP (1:800 dilution, Promega, Madison, WI); mouse monoclonal anti-clathrin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-caveolin1 (1:100, BD Transduction Laboratories, Franklin Lakes, NJ); mouse anti-EEA1 (1:300, BD Transduction Laboratories, Franklin Lakes, NJ); anti-LAMP1 (1:200, Stressgen Bioreagents, Ann Arbor, MI).

Secondary antibodies: Alexa Fluor 488-conjugated goat anti-Rabbit (1:1000 dilution, Molecular Probes, Eugene, OR); AlexaFluoro 647-conjugated goat anti-mouse (1:1000 dilution, Molecular Probes, Eugene, OR).

A fixable FM 1-43 (Molecular Probes, Eugene, OR) or a fixable version of FM 4-64 (FM 4-65, Biotium, Hayward, CA) was used in some experiments to view FM signal after immunocytochemistry. Images were assembled into figure palettes using Adobe Photoshop.

# CHAPTER 3. RESULTS

## 3.1 Rapid endocytosis in astrocytes

Endocytosis is one of the most fundamental processes in eukaryotic cells and has a number of important functions. It has long been considered as an indispensable housekeeping function of all animal cells (Cavalli et al., 2001).

To study astroglial endocytosis, a protocol modified from Noble's work (Jones et al., 1998) was used to obtain high quality pure astrocyte cultures (See Method). As an astroglial intermediate filament, GFAP (glial fibrillary acidic protein) is the most commonly used marker for identifying astrocytes *in vitro* (Song et al., 2002a). In our glia culture, > 95% of cultured cells are GFAP positive. Figure 1a shows a representative image of astrocytes stained by anti-GFAP antibody. We employed live fluorescence imaging and used FM 1-43, a styryl dye which has been widely used in neurons as a probe for studying synaptic vesicle recycling, to examine the basal endocytosis in glial cells and continuously monitored the movement of FM 1-43-labeled vesicles. Cultured astrocytes were stained with bath solution containing 20 μM FM 1-43. Surprisingly, following only 3 minutes of exposure to FM 1-43, the fluorescent dye has already been endocytosed by astrocytes, resulting in bright punctate staining pattern (Figure 1b, c).

We monitored the movement of FM 1-43 labeled vesicles by acquiring fluorescent images continuously and found that these vesicles show high dynamics (Movie 1). In addition, time-lapse imaging revealed that the majority of FM dye-labeled vesicles moved rapidly in the cytoplasm and gradually accumulated in the peri-nuclear area (Figure 1d). The rapid

movement of FM dye-labeled vesicles is dependent on microtubules, since depolymerizing microtubules with colchicine or nocodazole essentially abolished the vesicle movement (Movie 3). Thus, our live fluorescence imaging with FM dye revealed a rapid and constitutive endocytosis in cultured astrocytes.

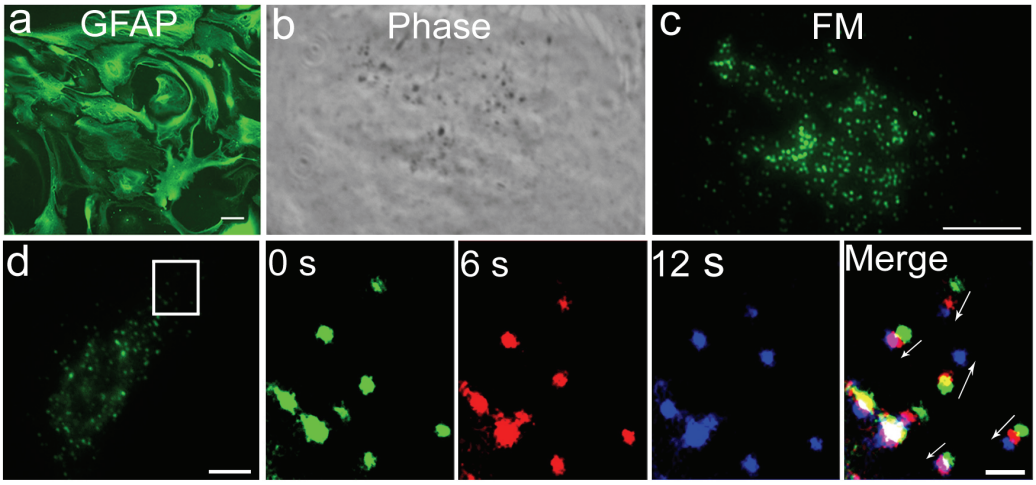


**Figure 1. Live imaging of constitutive endocytosis in cortical astrocytes.**

(**a**) A typical immunofluorescence image of cultured glial cells stained with anti-GFAP antibody. Almost all cells were GFAP-positive. (**b, c**) Representative images showing phase contrast image (**b**) and FM 1-43-labeled fluorescent puncta (**c**) in a cultured astrocyte. Astrocytes were incubated with FM 1-43 (20 μM) in bath solution for 5 min. (**d**) Time-lapse images illustrating dynamic movement of FM dye-labeled vesicles. FM dye-labeled puncta were accumulating in the perinuclear area. The color-coded images illustrate the location of FM dye-labeled vesicles at different time points: 0 s, 6 s, 12 s. The merged image showing a clear movement of vesicles toward the perinuclear area with one vesicle moving at opposite direction (arrows point to moving direction, from green to blue position). Scale bar in **a** is 50 μm, in **b-d** is 20 μm, and in the enlarged images of **d** is 2 μm.

## 3.2 FM dye is constitutively endocytosed by astrocytes.

Endocytosis could occur both constitutively and/or as a triggered response to extracellular signals. To investigate whether the rapid endocytosis is constitutive in astrocytes, we repeatedly loaded astrocytes with FM dye. Figure 2e show a schematic diagram illustrating the protocol used for multiple staining. Cells were stained with FM 1-43 for three rounds and for each round of staining, cells were loaded with 10 μM FM 1-43 for 4 minutes, then excessive dye were thoroughly washed out by the perfusion of bath solution and images within the same field were recorded. Figure 2a-c show representative samples of the same astrocytes repeatedly stained with FM for three times. Apparently an increased FM intensity was observed after each round of staining. Quantitative analysis confirms that fluorescence intensity within cells was greatly enhanced after repeated staining, which indicates that an additional amount of FM dye has been uptaken (Figure. 2d, n=6).

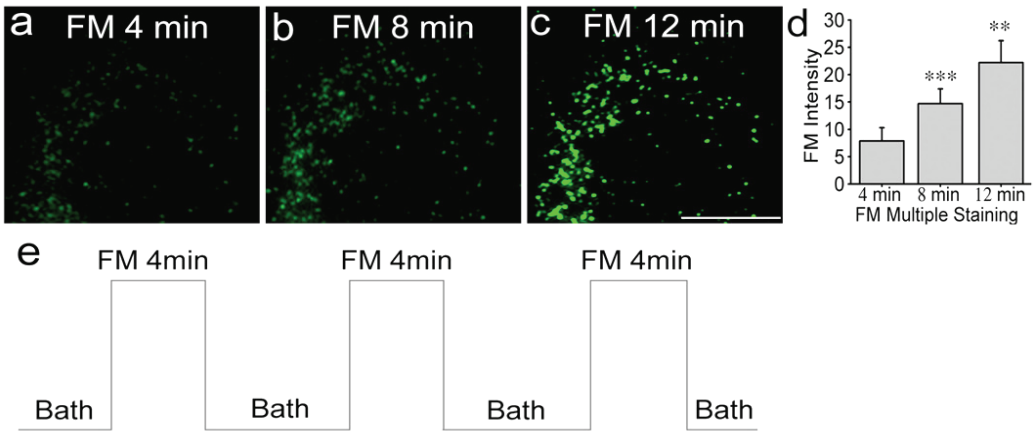


**Figure 2. FM dye is constitutively endocytosed by astrocytes.**

(**a-c**). Astrocytes were exposed to FM 1-43 for 3 consecutive rounds for live staining, with 10 min interval between each round of staining. (**a**) After first round of staining. (**b**) After second round of staining. (**c**) After third round of staining. (**d**) Quantitative changes of the average fluorescence intensity of astrocytes after each round of FM staining (n=6; ***, $p<0.001$; **, $p<0.002$; Student's *t* test between two consecutive staining). (**e**) A schematic drawing illustrating the protocol used for multiple staining. Scale bar 20 μm.

## 3.3 Accumulation of endocytosed vesicles in the peri-nuclear area but not inside Golgi network.

After being endocytosed, we observed a gradual accumulation of FM dye in the peri-nuclear area (Figure 3). To track the destination of FM-labeled vesicles after endocytosis, we loaded astrocytes with FM 1-43 (10 μM) for 5 min and then monitored the FM signals at different time intervals following the initial loading. After 15 minutes following FM staining, the majority of FM dye has already been found around the peri-nuclear area (Figure 3a1 and a2). After 2 to 4 hours, FM signals were found accumulated in the peri-nuclear area. To test whether Golgi complex is a potential destination for FM-labeled vesicles, we transfected cells with EGFP-GalT, a Golgi complex marker, which is widely used to illuminate Golgi network (Mackenzie et al., 1999). Two days after transfection, astrocytes were stained in bath solution containing 20 μM FM 4-64 (a FM 1-43 derivative, red-shifted dye) for 5 min and then incubated for 4 hrs to allow FM dye to accumulate in the peri-nuclear area. Simultaneous imaging of FM 4-64 (Figure 3b1 and b2, red) and EGFP-GalT (green) signals revealed a similar structure in the peri-nuclear area (Figure 3b1 and b2, merged panels; n=10). However, most punctate FM signals were not colocalized with the Golgi network.

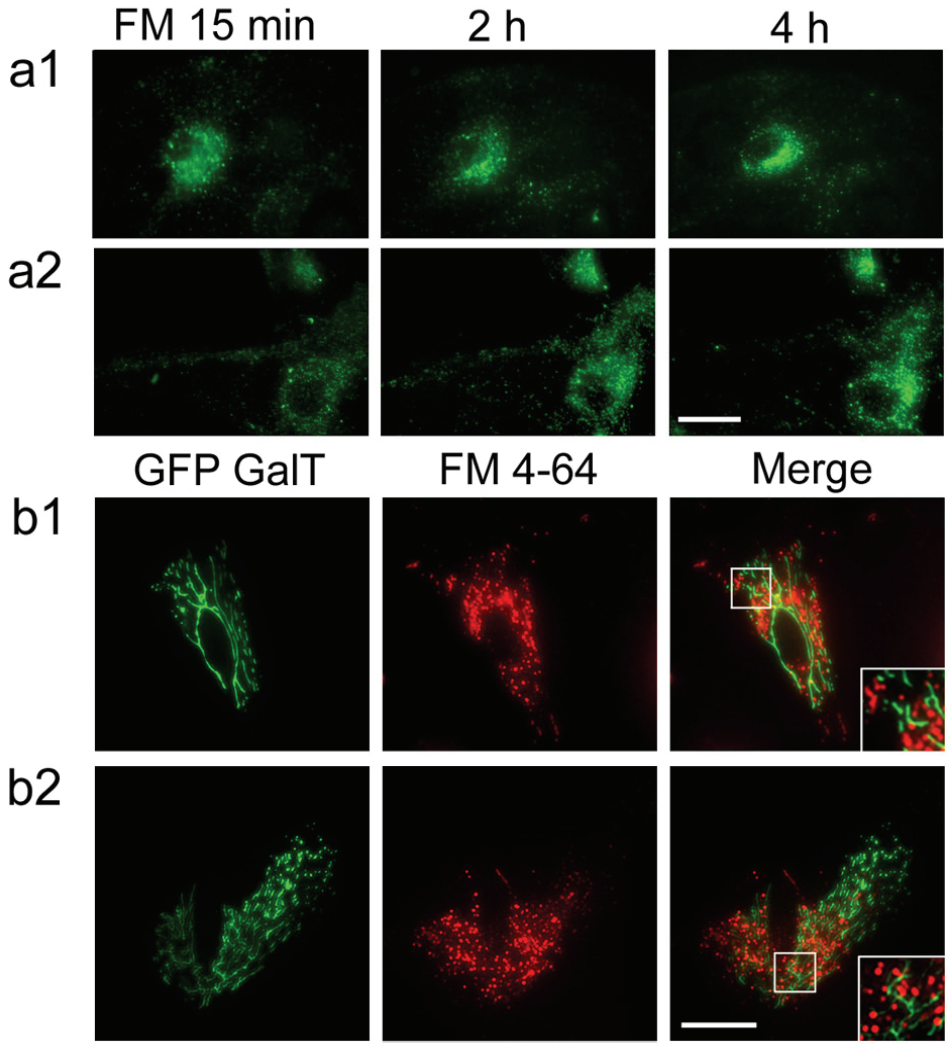


**Figure 3. Accumulation of endocytosed vesicles in the peri-nuclear area but not inside Golgi network.**

(**a1, a2**) Two representative examples showing that after endocytosis, FM dye accumulated around the peri-nuclear area. Cells were stained with FM 1-43 (10 μM) for 5 min and images of the same field were taken at different time intervals (15 min, 2 hr and 4 hr) after staining. (**b1, b2**) Two representative astrocytes with Golgi network illuminated by the expression of GFP-GalT and live staining with FM 4-64. Note that FM signal resides in punctate vesicles adjacent to but not colocalizing with the Golgi network. Scale bars, 50 μm.

## 3.4 The uptake of FM dye is faster than the uptake of transferrin in astroctyes

Among all endocytic events, clathrin-mediated endocytosis is the major form of endocytosis which is involved in the recycling of cell-surface receptors, such as transferrin receptors (Mousavi et al., 2004). It has been well established that the uptake of transferrin in mammalian cells is clathrin-mediated (Mellman, 1996). To determine whether the FM dye-labeled endocytosis in astrocytes is clathrin-mediated, we first compared it with the typical clathrin-mediated endocytic pathway labeled by Alexa Fluor 647-conjugated transferrin. Interestingly, when stained with FM 1-43 (20 μM) and Alexa Fluor 647-conjugated transferrin (200 μg/ml) simultaneously for 3 min, astrocytes were stained with significant FM signal (Figure 4a, n=12). In contrast, no detectable fluorescent transferrin signal was observed in the same cells (Figure 4b, n=12). After increasing the loading time to 30 min, which is typical for transferrin loading, we did observe bright staining of transferrin puncta in astrocytes (Figure 4c, n=12). These results suggested that in astrocytes the internalization of transferrin is a relatively slow process in comparison to the FM dye-labeled endocytosis. Indeed, even a brief exposure to FM dye, such as 30 sec incubation, was sufficient for astrocytes to uptake enough FM dye which can be detected under the fluorescent microscopy (Figure 4d, n=12).

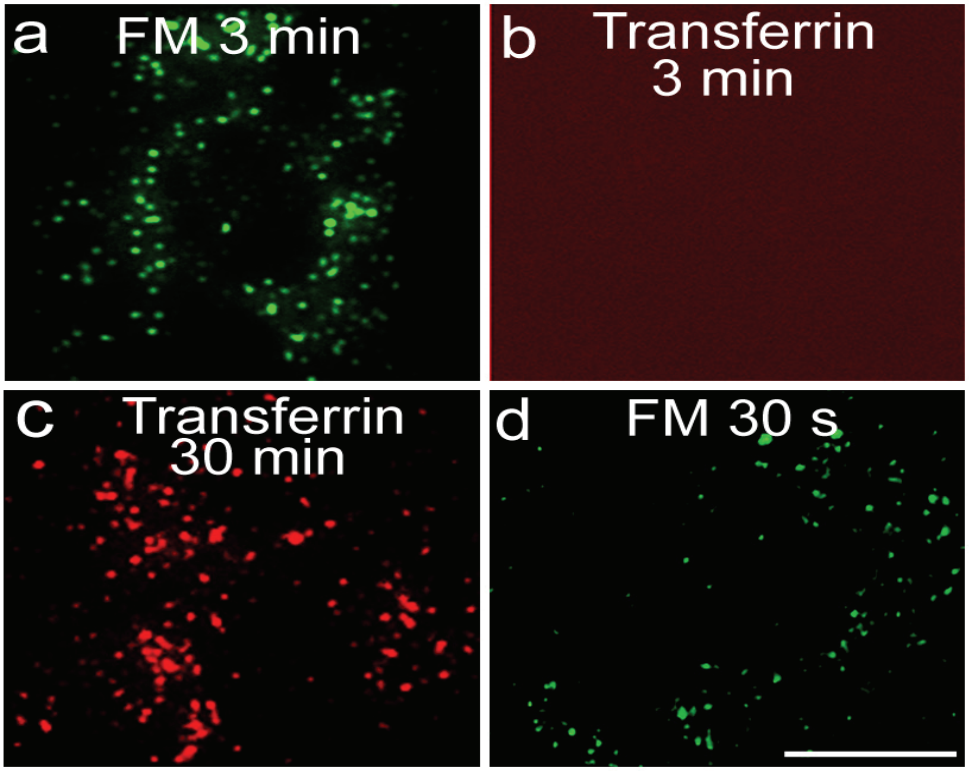


**Figure 4. FM dye was quickly uptaken by astrocytes.**

(**a, b**) Fluorescence images of an astrocyte stained with a mixture of FM 1-43 (**a**, 20 μM) and transferrin (**b**, Alexa Fluor 647-conjugated, 200 μg/ml) for 3 min. FM puncta are clearly visible but transferrin signal can not be detected for such short exposure in astrocytes. (**c**) Prolonged incubation of astrocytes with transferrin for 30 min resulted in detectable fluorescent puncta. (**d**) A brief exposure of astrocytes to FM 1-43 (40 μM) for 30 s is sufficient to label some vesicles, indicating a rapid endocytosis. Scale bar, 20 μm.

## 3.5 FM dye and transferrin are taken up via different endocytic pathways.

To further examine whether FM dye and transferrin are endocytosed through the same endocytic pathway, we conducted a sequential staining with FM 1-43 and transferrin. Firstly, we started with FM staining for 3 min, and then stained the same cells with Alexa Fluor 647-conjugated transferrin for 30 min (Figure 5 a-c, n=7). After sequential staining, cells were quickly fixed with fixation solution containing 4% paraformaldehyde to arrest any movement of endocytosed vesicles. Most FM dye were found accumulating around peri-nuclear area (Figure 5a) because of a >30 min time delay after initial endocytosis, which is consistent with data shown in Figure 3. In contrast, the transferrin signals show a distinctive pattern from that of FM staining. Only a small proportion of transferrin signals also accumulated at the peri-nuclear area, many transferrin-labeled vesicles were distributed throughout the cell (Figure 5b). An overlay of both FM and transferrin images (Figure 5c) and especially the enlarged image (Figure 5d) show a clear separation of the two signals from each other. Next, we reversed the order of the sequential staining by first incubating astrocytes with transferrin for 30 min and then stained the same cells with FM dye for 3 min. Transferrin was again distributed throughout the astrocytes (Figure 5e). The FM signal was now also widely distributed owing to an immediate fixation after a brief exposure to FM dye (Figure 5f). However, when overlaying FM and transferrin images together, these two signals were still mostly separated from each other (Figure 5g & h, n=7). Therefore, FM-labeled endocytic pathway appears to differ significantly from the classical transferrin-labeled endocytosis.

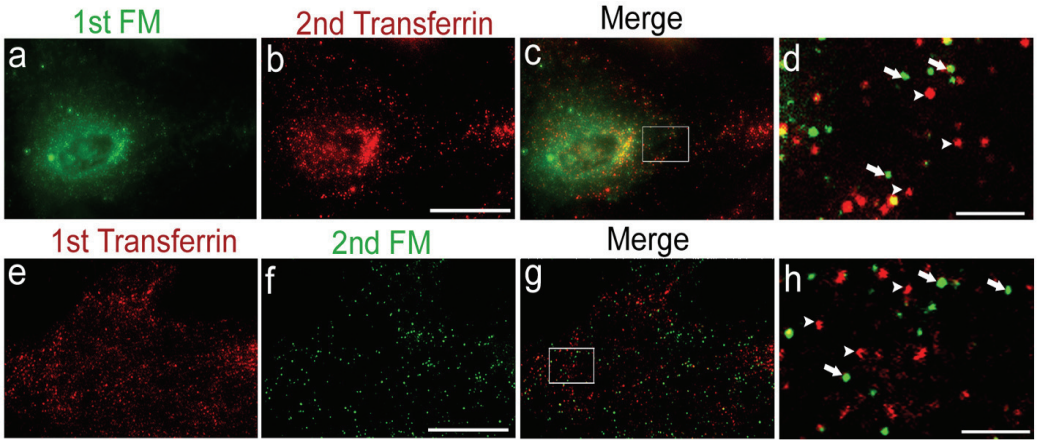


**Figure 5. FM dye and transferrin are taken via different endocytic pathways.**

Fluorescence images of the same cells sequentially stained first with FM 1-43 (3 min, **a**) and then transferrin (30 min, **b**). Images were taken after fixation to prevent vesicle movement. **(c)** Overlaid images of both FM (green) and transferrin signal (red). **(d)** Enlarged images from selected rectangle area in **(c)**. Note that the FM signal (green puncta, arrows) is largely separated from the transferrin signal (red puncta, arrowheads). **(e and f)** Fluorescence images of sequential staining of the same cells first with transferrin (30 min, e) and then FM 1-43 (3 min, f). **(g)** Merged picture showing the FM (green) and transferrin signal (red) in the same cells. **(h)** Enlarged image from selected area of **(g)** demonstrating that the FM signal (green puncta, arrows) is separated from transferrin signal (red puncta, arrowheads). Scale bar, 20 μM.

## 3.6 Rapid endocytosis in astrocytes is clathrin-independent.

To further investigate whether FM dye-labeled rapid endocytosis is clathrin-dependent, we treated astrocytes with hypertonic solution, 0.5 M sucrose, to block clathrin-mediated endocytosis (Heuser and Anderson, 1989). To confirm the effect of sucrose treatment on clathrin-mediated endocytosis, we loaded cells with Alexa Fluor 647-conjugated transferrin for 30 min. Indeed, the sucrose treatment largely blocked the internalization of transferrin and only fluorescent signal from background was defected (Figure 6b). In contrast, the sucrose treatment didn't show obvious effect on the endocytosis of FM dye. As shown in Figure 6c and d, the sucrose treated astrocytes (Figure 6d) uptake a similar amount of FM dye as those in control group (Figure 6c, quantification in Figure 6f). Without sucrose treatment, some FM dye can be internalized via clathrin-mediated endocytic pathway during the 3 min FM incubation. However, we did not see any significant increase of FM uptake in control group, indicating that only a very small portion of FM dye was endocytosed via clathrin-mediated endocytic pathway.

To more directly investigate the clathrin-mediated and FM dye-labeled endocytic pathways in astrocytes, two independent approaches were used to examine whether endocytosed FM dye was colocalized with clathrin. First, we transfected cells with EGFP-Clathrin light chain to label clathrin-coated vesicles and then stained cell with fixable FM 4-64. Astrocytes were briefly fixed right after FM loading and both FM (Figure 6h, red) and clathrin fluorescent signals (Figure 6g, green) were examined by confocal microscopy. Not surprisingly, as shown in the merged image (Figure 6i), FM-labeled red puncta were largely separated from clathrin signals, indicating FM dye were carried by vesicles without the coat of clathrin. Secondly, we performed live FM imaging followed by

immediate fixation and immunostaining with clathrin antibodies. As shown in Figure 6j-l, the fluorescent signals of fixable FM 1-43 (Figure 6j, green) and clathrin-antibodies (Figure 6k, red) show distinct staining patterns (Figure 6l, merged image), suggesting that they represent two different populations of vesicles. Together, these data suggest that FM dye-labeled rapid endocytosis in astrocytes is a clathrin-independent pathway.

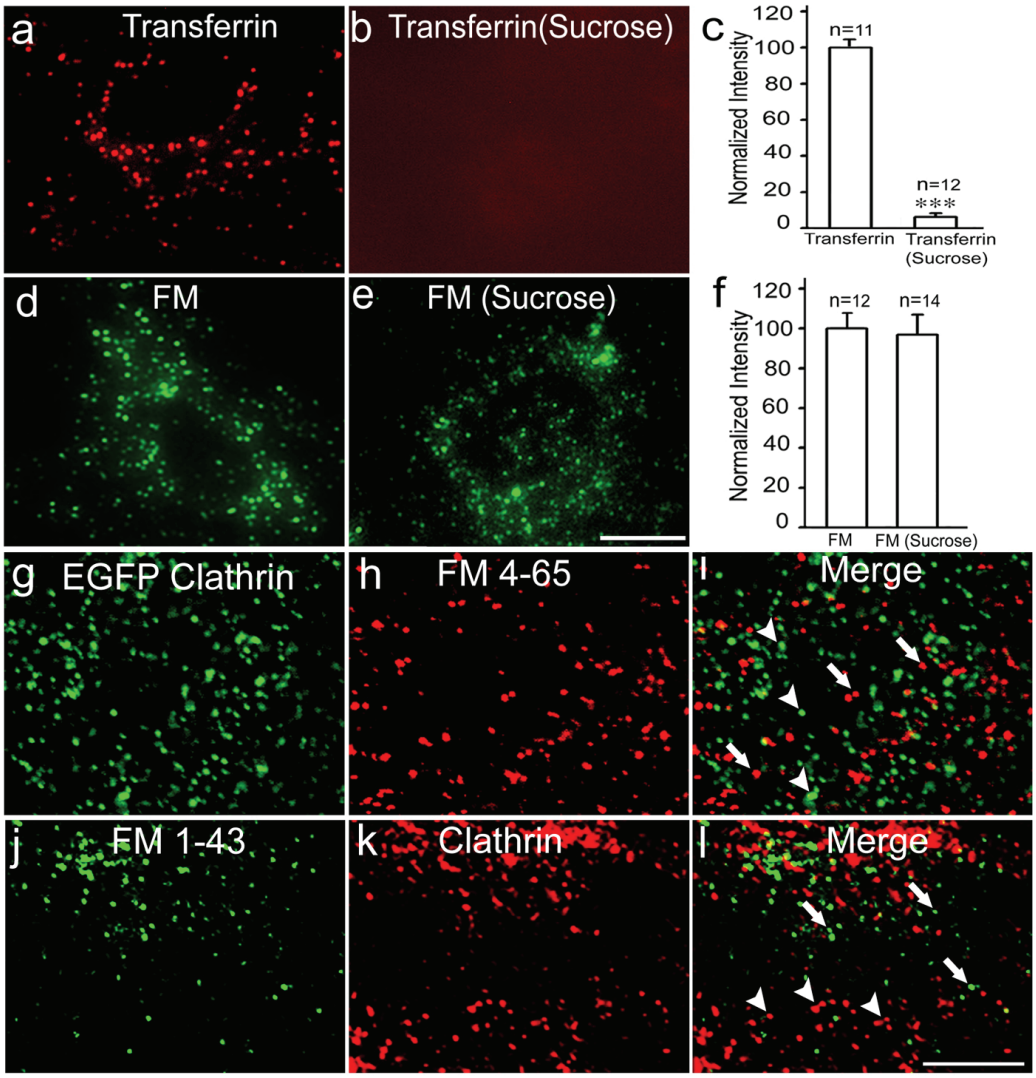


**Figure 6. Rapid endocytosis in astrocytes is clathrin-independent.**

**(a and b)** Fluorescence image of astrocytes incubated with transferrin (Alexa

Fluor 647-conjugated, 200 μg/ml) for 30 min with **(a)** or without **(b)** sucrose pretreatment (0.5 M, 30 min). Note that the uptake of transferrin was largely blocked with sucrose treatment. **(c)** Quantitative data showing the normalized fluorescent Alex-647 transferrin intensity in control and sucrose pretreated group (control, 1.00 ± 0.05; sucrose, 0.06±0.01, $p<0.0001$; Student'*s t* test). **(d)** Fluorescence image of control astrocytes stained with FM 1-43 (20 μM, 3 min). **(e)** FM image of astrocytes pretreated with sucrose (0.5 M, 30 min) to inhibit clathrin-mediated endocytosis. **(f)** Quantitative data showing the normalized FM intensity in control and sucrose pretreated group (control, 1.0 ± 0.1; sucrose, 0.97 ± 0.12, $p>0.8$; Student'*s t* test). **(g,h)** Fluorescent images showing the same cells overexpressing EGFP Clathrin light chain **(g)** were stained with FM 4-65 and fixed **(h)**. **(i)** Merged image showing FM puncta (red, arrows) are not localized with EGFP Caveolin1 (green, arrowheads). (**j, k**) Fluorescence images showing the same cells labeled by FM 1-43 (**j**) and anti-clathrin antibody (**k**). (**l**) Merged image showing that the FM puncta (green, arrows) are largely separated from the clathrin puncta (red, arrowheads). Scale bars, 20 μm.

## 3.7 Disrupting caveolae does not affect the rapid endocytosis.

Among non-clathrin dependent endocytic pathways, caveolae-dependent endocytosis is thought to play specific roles in cell signaling (Pelkmans and Helenius, 2002). In astrocytes, caveolae structures have been identified and are suggested to play an important role in cholesterol transportation (Cameron et al., 2002). We have indeed confirmed under electron microscopy that typical caveolae structures in omega shape aligned under plasma membranes in our cultured astrocytes (Figure 7a).

To test whether the internalization of FM dye is dependent on caveolae, we first treated astrocytes with filipin or methyl-β-cyclodextrin (MβCD), which are cholesterol-depleting drugs and widely used to abolish caveolae structures (Galvez et al., 2004; Yang et al., 2004). After filipin or MβCD treatment, astrocytes were stained with FM 1-43, 20 μM for 3 min. The drug treatment did not affect the uptake of FM in astrocytes. As shown in Figure 7c, d and e, the drug-treated astrocytes were capable of uptaking similar amount of FM dyes as cells without treatment (Figure 7b and e). These data indicate that drug treatment that abolishes caveolar structure does not affect the endocytosis of FM dye in astrocytes.

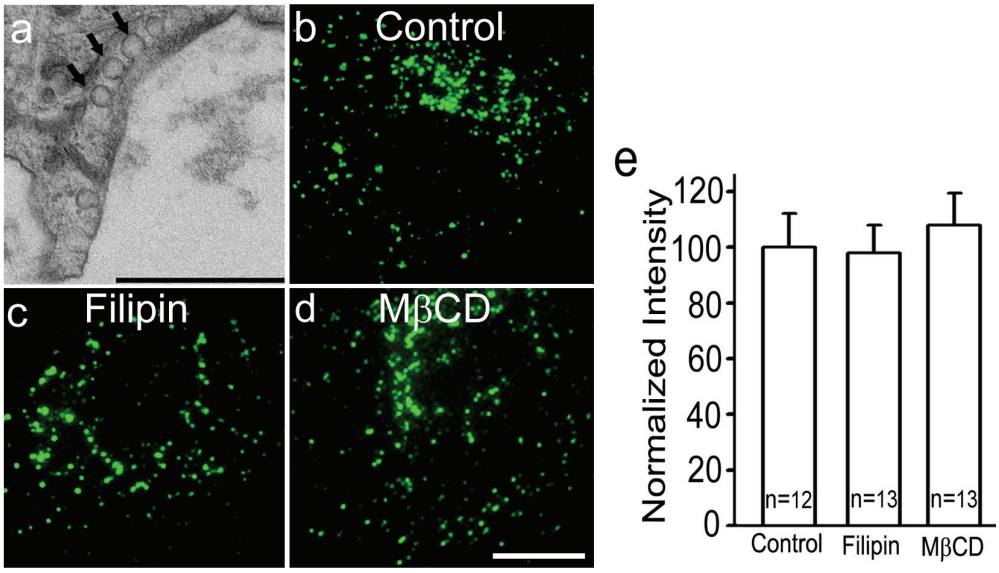


**Figure 7. Disrupting caveolae does not affect the rapid endocytosis.**

(**a**) Electron microscopic image showing clusters of caveolae (arrows) are aligned underneath the plasma membrane. Note the characteristic omega shape caveolae structures. Scale bar, 500 nm. (**b**) FM image of control cells (20 μM FM 1-43, 3 min). (**c, d**) FM images of cells pretreated with filipin (**c;** 10 μg/ml, 30 min) or MβCD (**d;** 10 mM, 15 min) to deplete membrane cholesterol and inhibit caveolae formation. The endocytosis of FM dye was not affected by filipin or MβCD treatment. **(e)** Normalized intensity of FM signal after drug treatments in comparison to the control group. (control, 1.0 ± 0.1; filipin, 0.98 ± 0.09, $p>0.8$; MβCD, 1.10 ± 0.12, $p>0.6$; Student's *t* test). Scale bar, 20 μm.

## 3.8 Astroglial rapid endocytosis is independent of caveolin

In addition to the pharmacological analysis, we transfected astrocytes with EGFP-caveolin1, which is widely used to illuminate caveolar structures (Kumari and Mayor, 2008). After transfection, cells were loaded with FM 4-65, a fixable version of FM 4-64 and fixed immediately after dye loading. By examining both the EGFP-caveolin1 (Figure 8a, green) and FM signals (Figure 8b, red), we found that the majority of FM labeled puncta were not overlaid with caveolin1 positive puncta (Figure 8c). Moreover, we performed live FM staining of astrocytes followed by retrospective immunostaining with caveolin1, which specifically labels caveolar structures (Figure 8d and e). From the merged image, we found that the majority of FM puncta (green) were distinctly separated from the caveolin1 puncta (red) (Figure 8f). These results suggest that the rapid endocytosis labeled by FM dye in astrocytes is caveolae-independent.

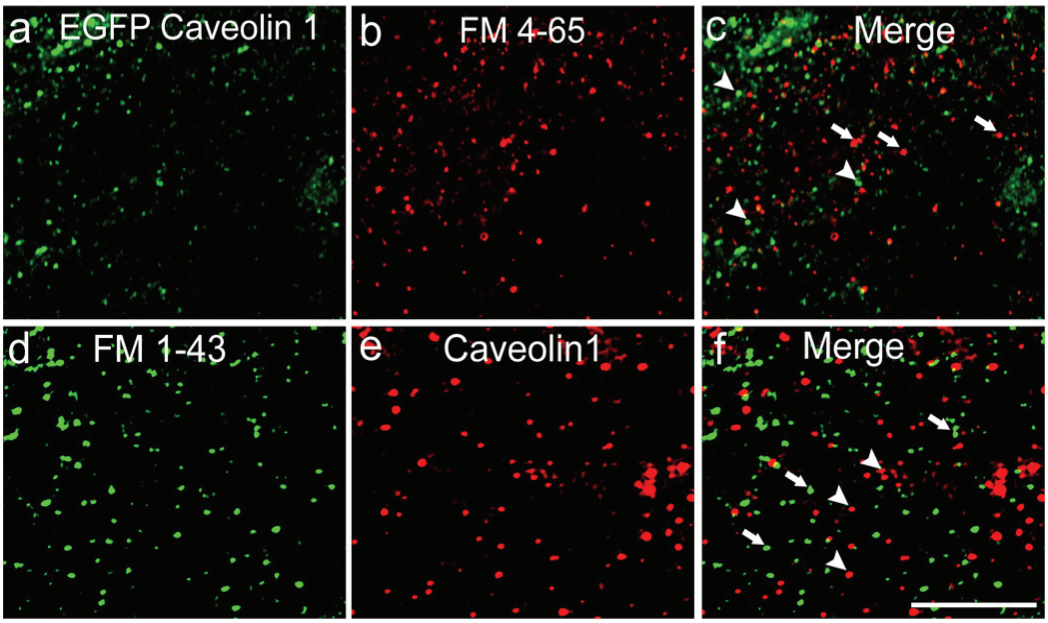


**Figure 8. Rapid endocytosis in astrocytes is caveolae-independent.** **(a, b)** Fluorescent images showing the same cells overexpressing EGFP Caveolin1 **(a)** were stained with FM 4-65 **(b)**. **(c)** Merged image showing FM puncta (red, arrows) are not localized with EGFP Caveolin1 (green, arrowheads). (**d, e**) Fluorescence images showing the same cells labeled by FM 1-43 (**d**) and caveolin1 antibody (**e**). (**f**) Merged image showing that the FM puncta (green, arrows) are largely separated from the caveolin1 puncta (red, arrowheads). Scale bars, 20 μm.

## 3.9 Astroglial rapid endocytosis is independent of dynamin

Dynamin, a member of superfamily of GTPases, is believed to be critical to the fission and internalization of both clathrin- and caveolin-coated endocytic vesicles (Oh et al., 1998; Sekiguchi et al., 1999). The overexpression of dynamin mutant (K44A) has been shown to significantly inhibit clathrin- and caveolin-mediated endocytosis, but dynamin-independent endocytosis has also been reported (Damke et al., 1994; Sekiguchi et al., 1999; Bonazzi et al., 2005). Therefore, we further tested whether the FM-labeled rapid endocytosis in astrocytes requires dynamin. Either wild type (WT) or a dominant-negative (K44A) form of dynamin was overexpressed in astrocytes for examining the effect on the endocytosis of FM dye or transferrin. Not surprisingly, when we overexpressed wild type (WT) and dynamin K44A in astrocytes, the uptake of transferrin was significantly inhibited by dynamin K44A (Figure 9a-d, quantified in e). In contrast, comparing to the expression of WT dynamin (Figure 9f and g), the mutant dynamin K44A did not block the FM-labeled rapid endocytosis (Figure 9h-i, arrow head, quantified data shown in j), suggesting that the FM-labeled rapid endocytosis in astrocytes is a novel endocytic pathway that is largely dynamin-independent. In addition, these results further backup our conclusions that the FM-labeled rapid endocytosis in astrocytes is not mediated through clathrin- or caveolin-coated vesicles because both of them are critically dependent on dynamin.

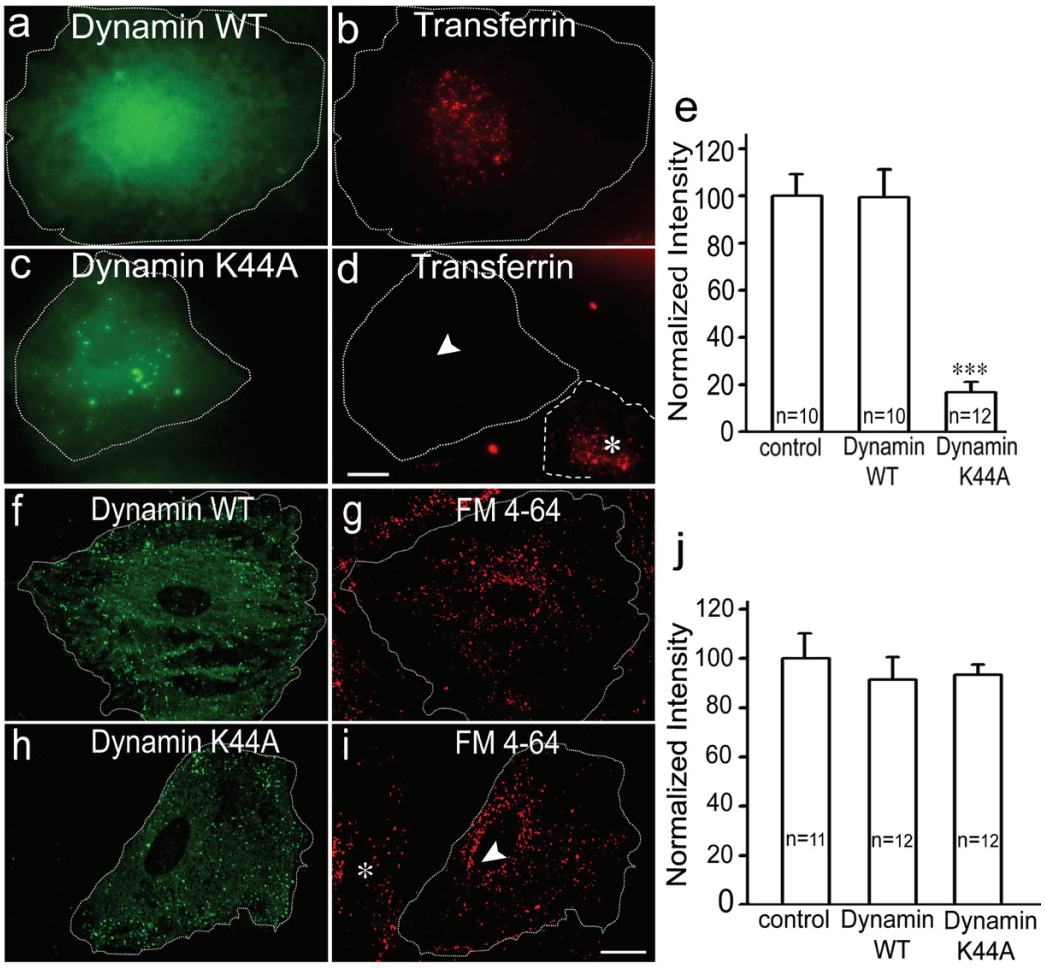


**Figure 9. Fast endocytosis in astrocytes does not require dynamin.**

**(a, b)** Fluorescence images of the same astrocyte overexpressing EGFP-tagged wild type dynamin (a) and then live stained with Alexa Fluor 647-conjugated transferrin (200 μg/ml, 30 min) **(b). (c, d)** EGFP **(d)** and transferrin **(d)** images of astrocytes overexpressing a dominant negative mutant dynamin K44A tagged with EGFP. The transferrin signal was significantly reduced in cells transfected with mutant dynamin K44A (arrowhead), compared to non-transfected neighbors (asterisk). **(e)** Quantitative data showing the normalized transferrin fluorescent intensity of different transfected groups in comparison to the non-transfected control cells (control, 1.00 ± 0.09; wild type dynamin, 0.99 ± 0.13, $p>0.9$;

dynamin K44A, 0.16 ± 0.04, p<0.0001; Student's *t* test). Scale bar, 20 μm. **(f, g)** Fluorescence images of GFP-dynamin WT **(f)** and FM 4-64 **(g)** in an astrocyte overexpressing GFP-WT dynamin. **(h, i)** Fluorescence images of GFP-dynamin K44A **(h)** and FM 4-64 **(i)** in an astrocyte overexpressing GFP-dynamin K44A. Both transfected (arrowhead) and non-transfected astrocytes (asterisk) showed clear FM staining. Dotted lines delineate transfected astrocytes. **(j)** Quantitative data showing the normalized FM 4-64 fluorescent intensity of different transfected groups in comparison to the non-transfected control cells (dynamin WT, 89 ± 9%, p>0.4; dynamin K44A, 91 ± 5%, p>0.5; Student's *t* test). Scale bar, 20 μm.

## 3.10 Rab5 regulates clathrin-mediated endocytosis

Rab5, a small GTPase, is shown to be critical to the formation and transportation of internalized vesicles. It has been proposed that when clathrin is transported to the vesicle membrane, Rab5 is recruited to the membrane to promote the membrane fission. This may also explain why the overexpression of dominant negative Rab5 can disrupt the clathrin-mediated endocytosis (Bucci et al., 1992; McLauchlan et al., 1998). To confirm the effect of Rab5 on transferrin endocytosis, cells were transfected with either wild type Rab5 or Rab5S34N, a dominant negative form of Rab5. The transfected cells were then loaded with Alexa Fluor 647-conjugated transferrin. Not surprisingly, as in the control experiment, Rab5 WT didn't show adverse effects on the internalization of transferrin (Figure 10a and b). However, in cells overexpressing dominant negative Rab5 (Figure 10c), the transferrin internalization was largely abolished (Figure 10d). These data confirm that the mutant dynamin indeed blocks the endocytosis of transferrin.

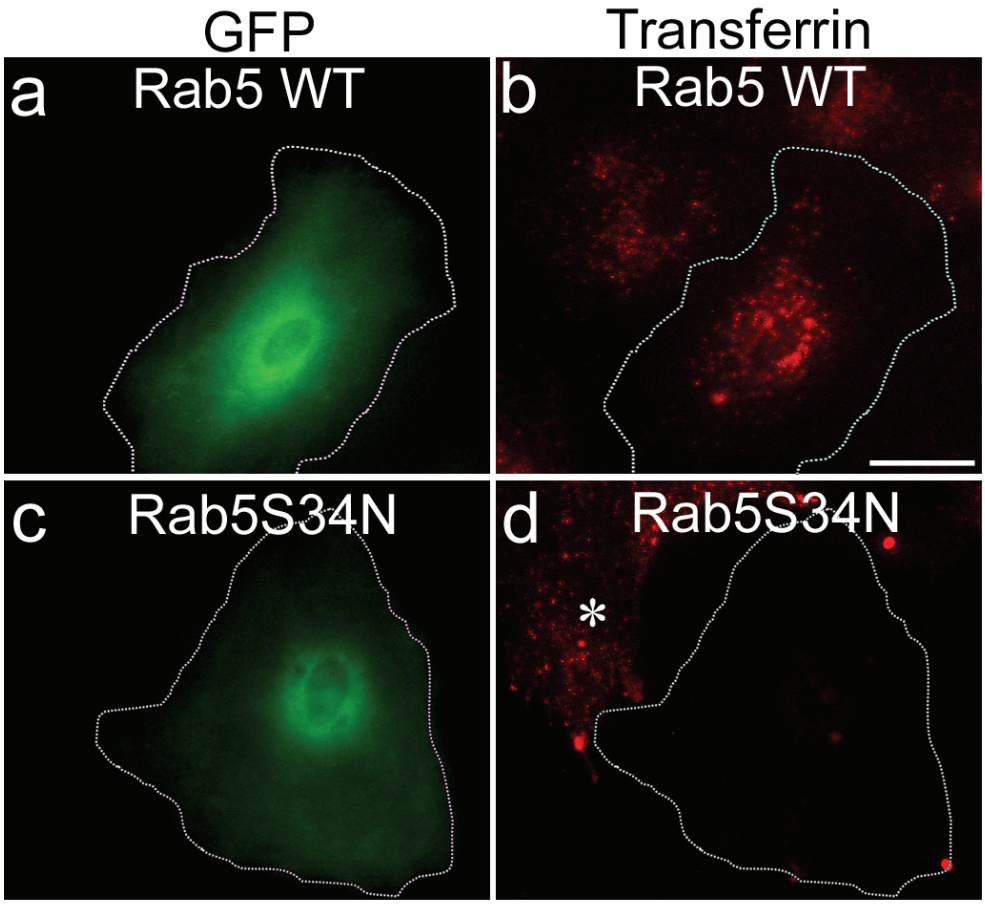


**Figure 10. Rab5 regulates clathrin-mediated endocytosis in astrocytes.** (**a, b**) Live fluorescent images of GFP (**a**) and transferrin signals (**b**) in astrocytes. Cells overexpressing wild type GFP-Rab5 showed normal transferrin uptake. Dotted line delineates the transfected cell. (**c, d**) Astrocytes transfected with a dominant negative mutant GFP-Rab5 S34N (**c**) showed a significant decrease of transferrin uptake (**d**) in comparison with non-transfected neighbors (asterisk). Scale bar, 20 μm.

## 3.11 Rab5 regulates rapid endocytosis

To test whether Rab5 regulates the FM labeled endocytosis in astrocytes, cells overexpressing WT Rab5 or Rab5S34N were loaded with FM dye. Rab5 WT didn't affect the uptake the FM dye (Figure 11a, b and e). In contrast, the overexpression of dominant negative form of Rab5, Rab5S34N, significantly inhibited the uptake of FM dye in comparison to the non-transfected astrocytes (asterisk) and Rab5 WT transfected cells (Figure 11c, d and quantified data shown in e; n=13, $p<0.0001$). These data indicate that the internalization of FM dye can be regulated by Rab5.

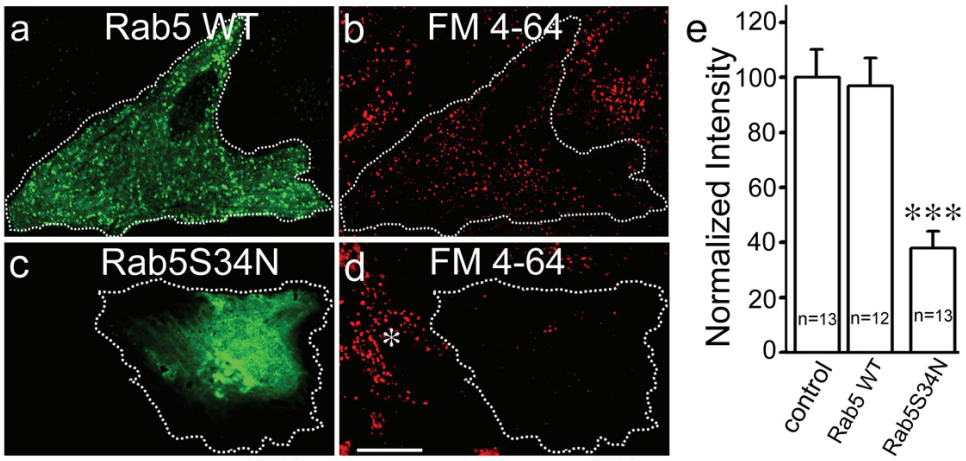


**Figure 11. Rapid endocytosis in astrocytes is regulated by Rab5.**
(**a, b**) Live fluorescent images showing that astrocytes overexpressing wild type GFP-Rab5 (**a**) had normal uptake of FM 4-64 (**b**). (**c, d**) Astrocytes transfected with a dominant negative mutant GFP-Rab5S34N (**c**) showed a significant decrease of FM dye uptake (**d**), in comparison with non-transfected neighboring cells (asterisk). Scale bar 20 μm. (**e**) Quantitative data showing the normalized fluorescent FM 4-64 intensity of different mutant Rab5 transfected groups in comparison to the nontransfected cells (control). (control, 1.0 ± 0.1; wild type Rab5, 0.97 ± 0.10, $p>0.8$; Rab5S34N 0.38 ± 0.06, $p<0.0001$, Student's *t* test).

## 3.12 Overexpression of Rab5S34N disrupts early endosomes

In mammalian cells, membrane-internalized vesicles are often first transported to early endosomes where cargos will be sorted before further delivered into other organelles such as late endosomes or Golgi complex (Cavalli et al., 2001; Sorkin and Von Zastrow, 2002). It has been well established that Rab5 plays an essential role in the biogenesis of early endosomes. In our experiments, wild type Rab5 and dominant negative mutant of Rab5 (Rab5S34N) were used to transfect cultured astrocytes. To confirm the effects of the Rab5 mutant, we performed immunostaining and labeled early endosomes by using antibody that recognizes EEA1, an early endosome specific protein. Indeed, in cells overexpressing EGFP Rab5S34N (Figure 12c, green), there were no obvious florescent signals of EEA1 (Figure 12d, red). In contrast, the neighboring non-transfected cell showed bright red puncta indicating the presence of undisrupted early endosomes (Figure 12d, asterisk). At the same time, the overexpression of Rab5 WT seemed to have no obvious adverse effect on the formation of early endosomes (Figure 12a and b).

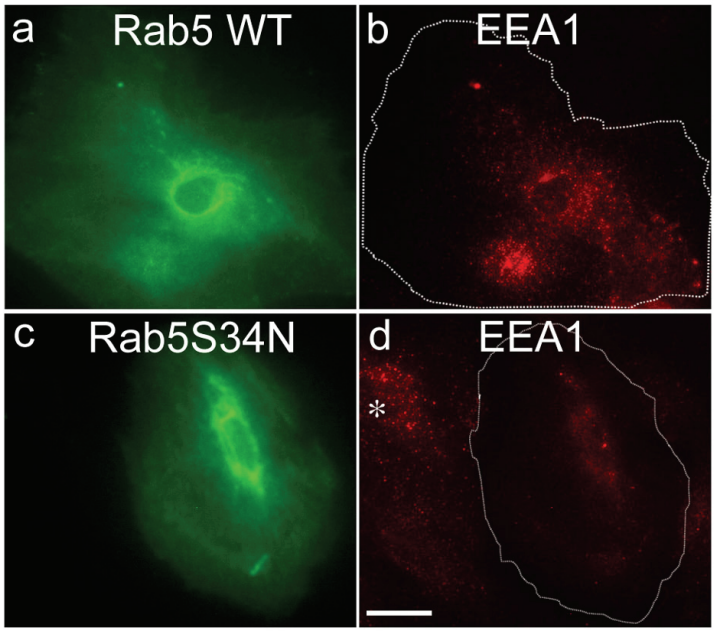


**Figure12. Rab5 regulates the formation of early endosomes.**

(**a, b**) Live fluorescent images showing that astrocytes overexpressing wild type GFP-Rab5 (**a**) had normal distribution of early endosomes labeled by anti-EEA1 antibody (**b**). (**c, d**) Astrocytes transfected with a dominant negative mutant GFP-Rab5S34N (**c**) showed a significant decrease in the number of EEA1 fluorescent puncta (**d**) in comparison with non-transfected neighboring cells (asterisk). Scale bar 20 μm.

## 3.13 FM dye accumulates in Rab5 Q79L-enlarged early endosomes

To further investigate the early endosomal regulation of the rapid endocytosis, we transfected cells with a constitutively active form of Rab5, Rab5Q79L. The overexpression of Rab5Q79L resulted in the appearance of enlarged early endosomes because the high activity of Rab5Q79L greatly enhances homotypic endosomal fusion (Figure13a, arrows), which is consistent with previous research (Roberts et al., 1999; Rink et al., 2005).

Interestingly, after staining Rab5Q79L-transfected cells with FM dye, we found that a large amount of FM dye accumulated in the enlarged early endosomes labeled by EGFP Rab5Q79L (Figure 13b and c). To confirm that the Rab5Q79L-enlarged vesicles are indeed early endosomes, we performed immunostaining with early endosome marker EEA1, and found that the florescent signals of Rab5Q79L (Figure 13d, green) were well colocalized with EEA1 (Figure 13e, red and Figure 13f, merge). Furthermore, live staining with fixable FM 1-43 followed by immediate fixation and retrospective immunostaining with EAA1 showed a nice colocalization between FM and EEA1 puncta in astrocytes (Figure 13g-i). These results suggest that after being internalized, the FM dye-labeled rapidly endocytosed vesicles are first sorted through early endosomes, like classical endocytic pathways.

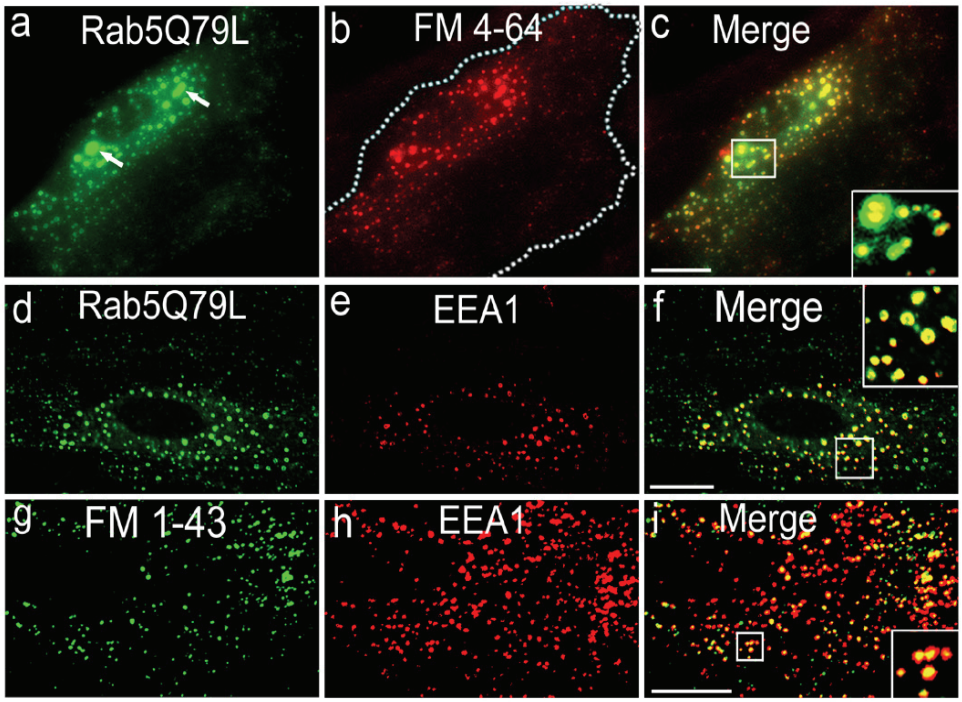


**Figure 13. FM dye accumulate in Rab5 Q79L-enlarged early endosomes.**

(**a, b**) GFP image (**a**) and FM 4-64 staining (**b**) of astrocytes overexpressing a constitutively active form GFP-Rab5Q79L. Note the large green puncta after overexpressing GFP-Rab5Q79L (arrows in **a**). (**c**) Merged image showing that the FM puncta (red) are well colocalized with Rab5Q79L-enlarged vesicles (green). (**d–f**) Fluorescent images of an astrocyte overexpressing GFP-Rab5Q79L (**d**) and immunolabeled by early endosome marker EEA1 (**e**). Merged image (**f**) showing that Rab5Q79L-enlarged vesicles are well colocalized with EEA1 puncta. (**g–i**) Fluorescent images of astrocytes labeled with fixable FM 1-43 (**g**) and EEA1 antibodies (**h**). Cells were fixed immediately after FM staining and images were taken simultaneously after immunostaining. (**i**) showed nice co-localization between FM 1-43 and EEA1 puncta, indicating that FM dye was endocytosed into early endosomes. Scale bars, 20 μm.

## 3.14 FM dye quickly accumulates in late endosomes/lysosomes.

It is well known that some cargos after internalization are transported to lysosomes where they will be degraded. To investigate whether FM dye is transported to lysosomes and how long it will take for FM dye to accumulate in lysosomes, we perform pulse-chase experiments. To do that, astrocytes were stained with fixable FM 1-43 and then washed for 2, 6, 15 or 120 min before fixed thus the transportation of endocytosed FM dye were stopped at different time points we assigned. The fixed cells were further immunostained with LAMP1, a specific marker for late endosomes/lysosomes (Figure 14).

For cells fixed after a 2 min washing, FM and LAMP1 signals were clearly separated (Figure 14a-c), indicating that at this stage immediate after internalization, FM dye may still remain in early endosomes (as shown in Figure 13h-i). However, after 6 min of washing before fixation, some FM dye-labeled puncta were already colocalizing with LAMP1 puncta (Figure 14d-f), suggesting that FM dye-labeled vesicles can fuse with or mature into late endosomes/lysosomes within a few minutes following endocytosis. With a 15 min washing before fixation, the majority of FM signal was found accumulated in late endosomes/lysosomes (Figure 14g-i). Upon 2 h, almost all FM signal was well overlaid with LAMP1 staining (Figure 14j-l). Based on these findings, we conclude that after internalization, FM dye can be quickly transported to late endosomes/lysosomes and gradually accumulate there. This further suggest that astroglial cells can rapidly endocytose and degrade or store extracellular molecules.

In addition, we found that in astrocytes overexpressing Rab5Q79L, the trafficking of FM dye seemed to slow down. After 48 hrs of transfection, we incubated live astrocytes with FM 4-65 (fixable FM 4-64, 5 min) and washed for more than 15 min before fixation and immunostaining with LAMP1. The fluorescent signals of EGFP Rab5Q79L (green), FM 4-65(blue) and LAMP1 (red)

were examined under a confocal microscopy. As we expected, the Rab5Q79L-enlarged early endosomes (Figure 14m, green) hardly colocalized with LAMP1-labeled late endosomes/lysosomes (Figure 14n, red), as shown by lacking yellow signal in the merged image (Figure14p, merge). However, in the Rab5Q79L-transfected cell, some FM dye still accumulated inside the enlarged early endosomes while others were colocalized with LAMP1-labeled late endosomes/lysosomes (Figure 14o, p). As we showed before, 15 min after dye loading, the majority of dye was already accumulating in late endosomes/lysosomes (Figure 14g-i). But for the same time interval, in astrocytes overexpressing the constitutively active form of Rab5, the enlarged Rab5Q79L positive early endosomes still hold a large portion of FM dye. Such a delay in the exit of FM dye from enlarged early endosomes supports that the transition from early endosomes to late endosomes/lysosomes is tightly regulated by Rab5, more specifically, a rapid conversion of Rab5-GTP into Rab5-GDP (Zerial and McBride, 2001).

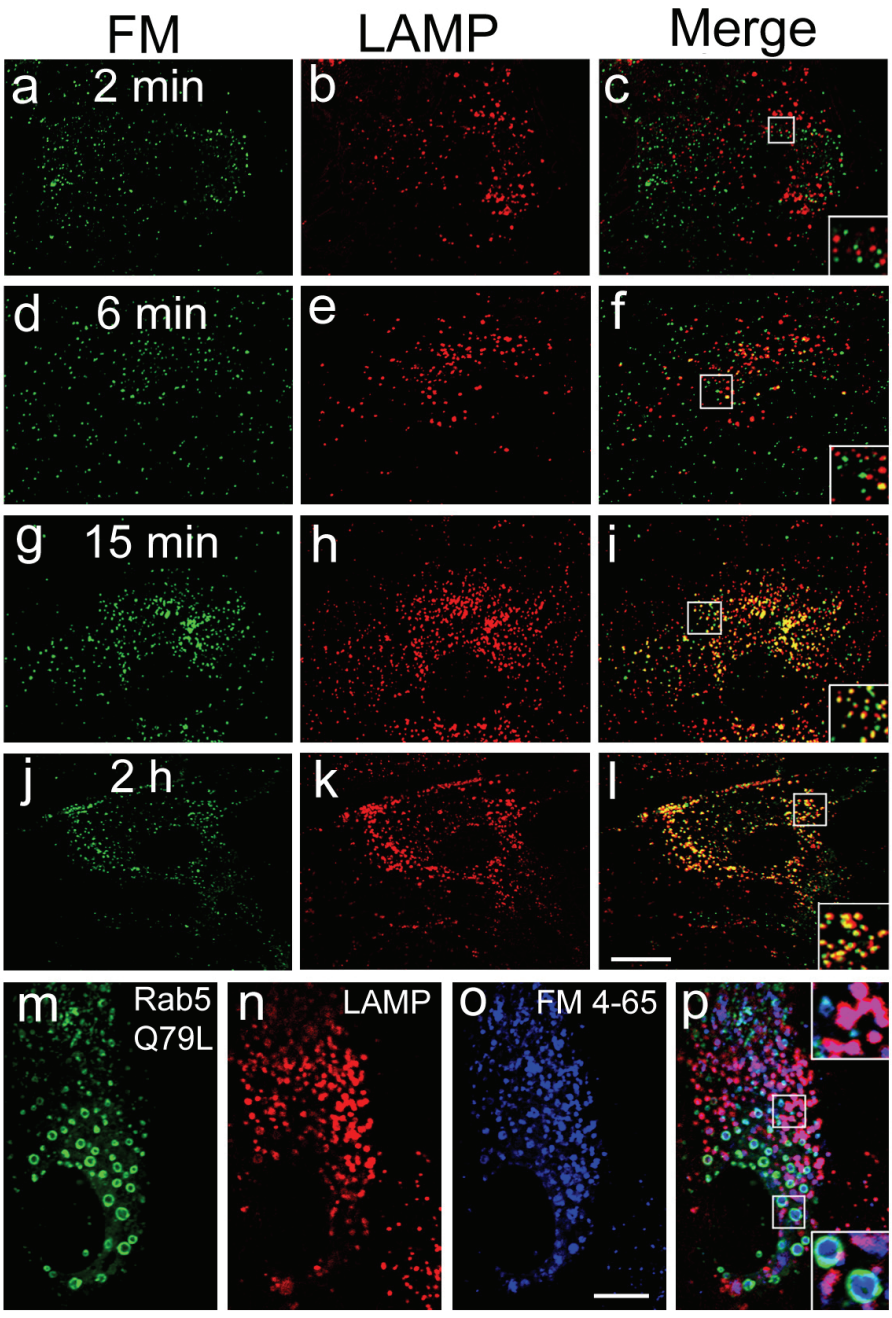


**Figure14. FM dye quickly accumulates in late endosomes/lysosomes.**

(**a-c**) Fluorescent images of astrocytes first live stained with fixable FM 1-43 (**a**) and then quickly fixed for immunostaining with the late endosome/lysosome marker LAMP1 (**b**). The 2 min indicates the time interval between FM staining and fixation. (**c**) Merged image showing that following immediate endocytosis,

the majority of FM puncta (green) are not yet in late endosomes/lysosomes (red). Inset in the lower corner showing enlarged view. (**d-f**) When the time interval was increased to 6 min between FM staining (**d**) and fixation for LAMP1 immunostaining (**e**), some FM puncta showed colocalization with LAMP1 (**f**, yellow puncta). (**g-i**) After a 15 min interval between FM staining and fixation, the majority of FM puncta (**g**) were well colocalized with LAMP1 (**h**), as evident from the merged image (**i**). FM and LAMP images were taken simultaneously after immunostaining. (**j-l**) 2 hr after FM staining, almost all FM puncta (**j**) were overlapped with LAMP1 (**k**) as shown in the merged image (**l**). (**m-p**) Fluorescent images showing the colocalization of FM dye-labeled puncta with both early endosomes and late endosomes/lysosomes. (**m**) Enlarged early endosomes after the overexpression of GFP-Rab5Q79L. (**n**) Late endosomes/lysosomes labeled by LAMP1. (**o**) Fixable FM 4-65 staining. (**p**) Merged images of FM 4-65 (Blue), GFP-Rab5Q79L (green), and LAMP (red). Insets are enlarged images from boxed regions. Note that some FM dyes were accumulated inside the Rab5Q79L-enlarged early endosomes (lower inset), while others were colocalizing with LAMP1-labeled vesicles (upper corner). Scale bar, **a-l** 20 μm, **m-p** 10 μm.

## 3.15 Rapid endocytosis of ATP and accumulation in lysosomes

To investigate the function of FM dye-labeled rapid endocytosis in astroglial cells, we examined whether gliotransmitters such as ATP can be internalized through the same endocytic pathway. Astrocytes were incubated with bath solution containing both FM 1-43 and MANT-ATP, a fluorescent ATP analog, for 5 min. After staining, both FM signal and MANT-ATP fluorescences were recorded simultaneously. Interestingly, live fluorescent images showed a similar distribution pattern between FM dye (Figure 15a, green) and MANT-ATP (Figure 15b, red) signals. In addition, these signals were well colocalized with each other in the merged image (Figure 15c). Moreover, we have shown previously that FM dye after being internalized, quickly accumulated in late endosome/lysosomes. We further investigated whether ATP, like FM dye, will be deposited to lysosomes shortly following endocytosis. To achieve this, we incubated astrocytes with LysoTracker (100 nM, 30 min), a fluorescent dye that can specifically label lysosomes/late endosomes. After LysoTracker staining, cells were incubated with MANT-ATP for 5 min and followed with a 15 min washing in bath solution. We found that the LysoTracker and MANT-ATP signals were colocalized as shown in the merged image (Figure 15d-f), which suggested that like FM dye, ATP is also rapidly endocytosed and transported to late endosomes/lysosomes.

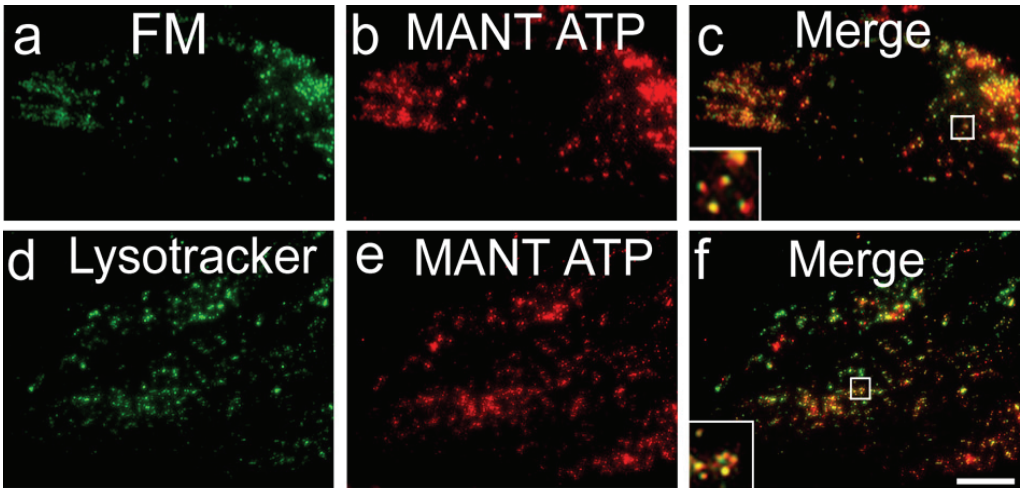


**Figure 15. Rapid endocytosis of ATP and accumulation in late endosomes/lysosomes.** (**a-c**) MANT-ATP and FM dye were endocytosed by astrocytes through the same pathway. Astrocytes incubated with MANT-ATP (300 μM) and FM 1-43 for 5 min showed the same pattern between FM (**a**) and MANT-ATP staining (**b**). Merged image (**c**) showing well-colocalized MANT-ATP (red) and FM puncta (green). (**d-f**) MANT-ATP is quickly transported to late endosomes/lysosomes. (**d**) Fluorescent image of Lysotracker staining. (**e**) MANT-ATP staining image. (**f**) Merged image showing that MANT-ATP puncta are well-colocalized with Lysotracker-labeled puncta. Scale bar, 20 μm

## 3.16 ATP and FM dye both taken with the rapid endocytic pathway

We have shown that FM dyes were endocytosed via a non-classical pathway, which is independent of dynamin. We further examined whether ATP is endocytosed through the same pathway. We overexpressed dominant negative dynamin K44A in astrocytes and then analyzed the endocytosis of MANT-ATP by incubating cells with MANT-ATP for 5 min. The expression of dynamin K44A seemed to have no effect on the uptake of MANT-ATP in astrocytes (Figure 16a, b). This suggested that like FM dye, ATP is endocytosed through a dynamin-independent pathway. In addition, our previous experiments demonstrated that FM-labeled vesicles are first sorted to early endosome. We then investigated whether ATP is also sorted through early endosomes after rapid endocytosis. Astrocytes were transfected with GFP-Rab5Q79L and two days later were stained with MANT-ATP (Figure 16c, d). Merged image showed that many MANT-ATP puncta colocalized with Rab5Q79L-enlarged early endosomes (Figure 16e, arrows). At the same time, some MANT-ATP molecules might have been transported to lysosomes (Figure 16e, arrowheads; compare with Figure 14p) and not accumulate in the Rab5Q79L positive early endosome anymore. These results suggest that the rapid endocytosis in astrocytes may play an important role in removing extracellular signaling molecules including gliotransmitters by rapidly transporting them to lysosomes for storage or degradation.

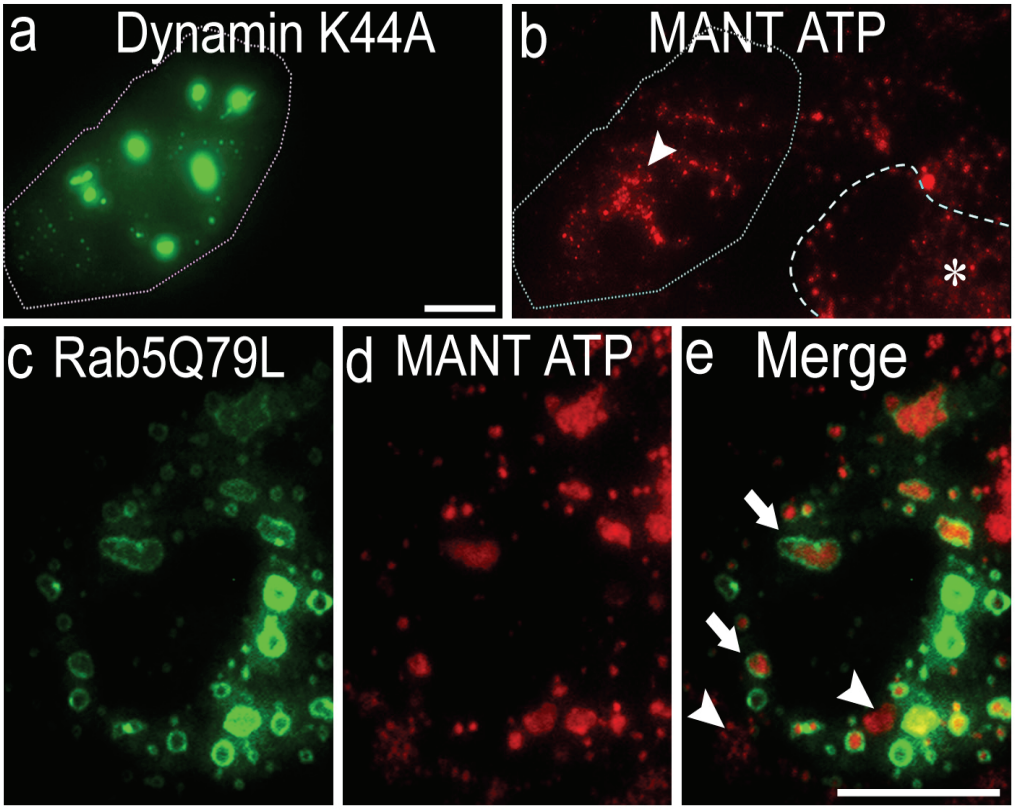


**Figure 16. ATP and FM dye both undergo the rapid endocytic pathway** (**a, b**) GFP image (**a**) and MANT-ATP staining (**b**) of astrocytes overexpressing the dominant negative Dynamin K44A. Both transfected (arrowhead) and non-transfected astrocytes (asterisk) showed similar MANT-ATP staining. (**c-e**) GFP (**c**) and MANT-ATP images (**d**) of astrocytes overexpressing a constitutively active form GFP-Rab5Q79L. Merged image (**e**) showing that some MANT-ATP puncta (red, arrows) are well-colocalized with Ραβ5Θ79Λ–ενλαργεδ ωεσιχλεσ (γρεεν), ωηιλε οτηερσ (αρροωηεαδσ) μ αψ ηαωε βεεν τρανσπορτεδ ουτσιδε οφ τηε εαρλψ ενδοσομεσ. Σχαλε βαρσ, 20 μm.

## 3.17 Rapid endocytosis in astrocytes is regulated by intracellular $Ca^{2+}$

Since the rapid endocytosis in astrocytes may function in internalizing signaling molecules, we next investigated how the rapid endocytosis in astrocytes is functionally regulated. We first tested whether the rapid endocytosis in astrocytes is dependent on extracellular $Ca^{2+}$, as it is important for synaptic vesicle cycling at nerve terminals. Astrocytes were incubated with FM 1-43 for 3 min in control (2 mM $Ca^{2+}$) or $Ca^{2+}$-free bath solution containing EGTA. Interestingly, extracellular $Ca^{2+}$ did not appear to affect the uptake of FM dyes in astrocytes (Figure 17a and b). The intracellular $Ca^{2+}$ oscillation in astrocytes is closely associated with astroglial functions (Fiacco and McCarthy, 2006). We then examined whether intracellular $Ca^{2+}$ might have any effect on the rapid endocytosis in astrocytes. A calcium ionophore A23187, which forms $Ca^{2+}$ channels on the plasma membrane to allow $Ca^{2+}$ influx, was used to increase the intracellular $Ca^{2+}$ concentration in normal bath solution. Remarkably, the FM-labeled rapid endocytosis in astrocytes was greatly enhanced with the treatment of A23187 (Figure 17c). In contrast, decreasing intracellular $Ca^{2+}$ by pretreatment with $Ca^{2+}$-chelator BAPTA-AM (25 μM, 45 min) significantly decreased the FM uptake by astrocytes (Figure 17d). Figure 17e show the quantification of the average FM intensity in astrocytes under different manipulations of extracellular or intracellular $Ca^{2+}$, clearly indicating that FM-labeled rapid endocytosis in astrocytes is regulated by intracellular $Ca^{2+}$ signal.

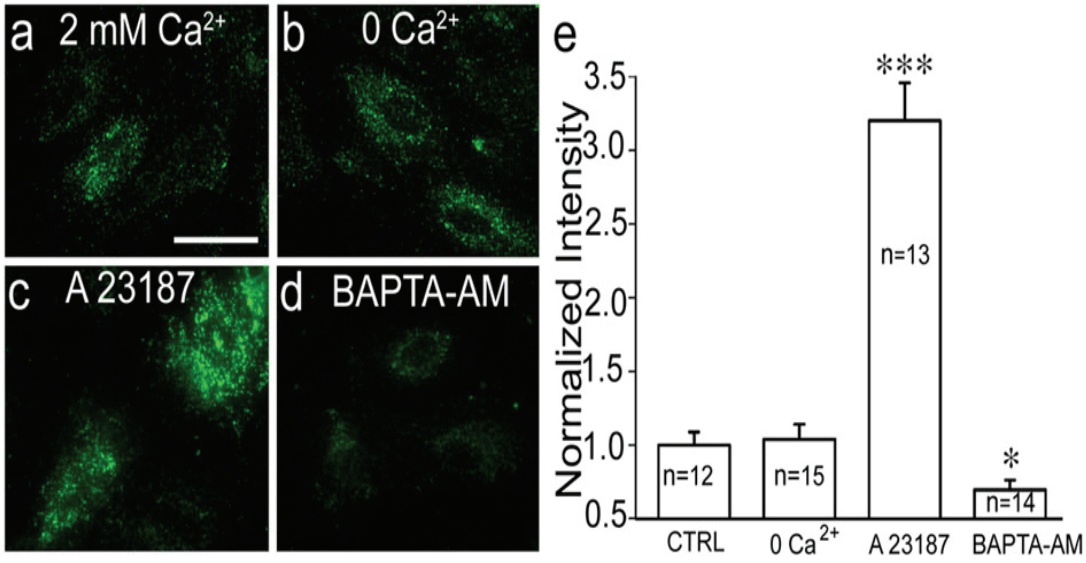


**Figure 17. Rapid endocytosis in astrocytes is regulated by intracellular $Ca^{2+}$.**

**(a)** FM image of control group. Cells were stained with 20 μM FM 1-43 for 3 min in normal bath solution containing 2 mM $Ca^{2+}$. **(b)** FM image of cells loaded with 20 μM FM 1-43 for 3 min in $Ca^{2+}$-free bath solution. **(c and d)** FM images of cells pretreated with A23187 (10 μM, 10 min; c) or BAPTA-AM (25 μM, 45 min; d). FM signal was enhanced after treatment with A23187, but decreased after BAPTA-AM treatment. Scale bar for a – d, 50 μm. **(e)** Normalized intensity of FM signal after different treatments in comparison to the control group (0 $Ca^{2+}$, 1.04 ± 0.10, $p>0.7$; A23187, 3.2 ± 0.3, $p<0.001$; BAPTA-AM, 0.70 ± 0.06, $p<0.02$; Student's *t* test).

## 3.18 Rapid endocytosis in astrocytes is regulated by ATP

The intracellular $Ca^{2+}$ concentration in astrocytes is known to be regulated by gliotransmitter ATP (Guthrie et al., 1999; Volterra and Meldolesi, 2005; Suadicani et al., 2006). Consistent with previous studies, we confirmed that acute application of ATP (10 μM, 30-50 sec) induced a significant increase of intracellular $Ca^{2+}$ in astrocytes (Figure 18e). We then test whether ATP-induced $Ca^{2+}$ increase may affect the rapid endocytosis. To do that, we treated astrocytes with FM 1-43 (20 μM, 90 sec) in the presence or absence of ATP (10 μM). Interestingly, ATP treatment greatly enhanced the endocytosis of FM dye compared to control (Figure 18a-b). Because ATP enhancement in some astrocytes was very strong, we had to reduce the FM loading time to 90 sec to prevent the saturation of FM signal under ATP treatment which gave less FM signal under control condition than the usual intensity after 3-5 min loading. To confirm that the ATP effect is mediated specifically by an increase of intracellular $Ca^{2+}$, we depleted intracellular $Ca^{2+}$ store with thapsigargin, a specific intracellular $Ca^{2+}$ pump inhibitor. Indeed, $Ca^{2+}$ imaging experiments verified that thapsigargin pretreatment (10 μM, 10 min) prevented the increase of intracellular $Ca^{2+}$ in astrocytes with ATP application (Figure 18f). We then pretreated astrocytes with thapsigargin before staining astrocytes with FM dye (20 μM, 90 sec) together with ATP (10 μM). After depleting intracellular $Ca^{2+}$ store, the application of ATP no longer increased the uptake of FM dye by astrocytes (Figure 18c-d). Based on these results, we conclude that the FM-labeled rapid endocytosis in astrocytes can be regulated by gliotransmitter ATP through intracellular $Ca^{2+}$ signaling.

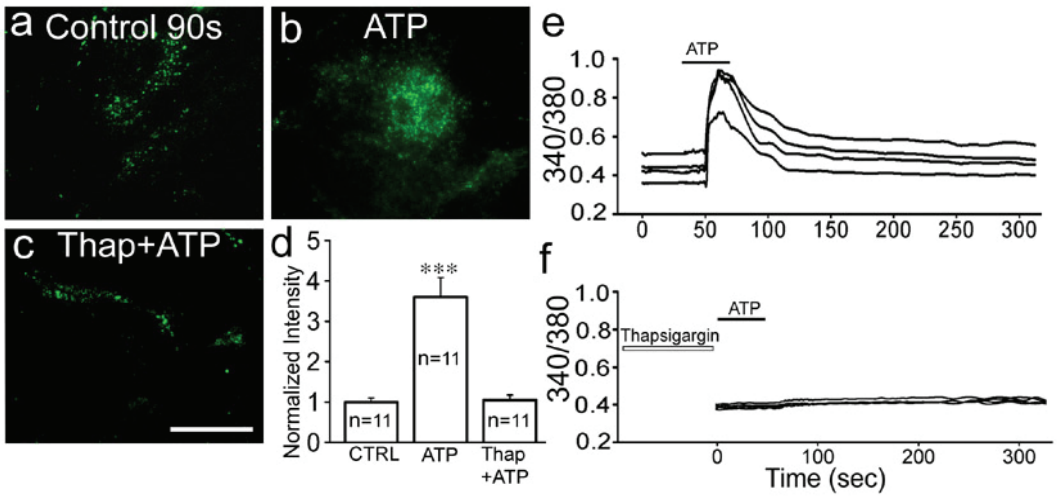


**Figure 18. Rapid endocytosis in astrocytes is regulated by ATP.**

**(a and b)** FM images of astroglial cells stained with FM 1-43 (20 μM) for 90 sec in $Ca^{2+}$-free bath solution without (a) or with ATP (10 μM, **b**). Note a substantial enhancement in FM signal in the presence of ATP. **(c)** FM image of cells pretreated with thapsigargin (10 μM, 10 min) to deplete internal $Ca^{2+}$ store and then loaded with FM 1-43 for 90 sec in the presence of ATP. No enhancement of the FM uptake was detected. **(d)** Normalized intensity of FM signal in different treatments. FM intensity was significantly increased after ATP treatment (3.6 ± 0.5, $p<0.001$, Student'*s t* test) comparing to controls, but this enhancement was abolished by pretreatment with thapsigargin (1.1 ± 0.1, $p>0.7$). **(e)** Representative recordings showing acute application of ATP (10 μM) induced a significant increase of intracellular $Ca^{2+}$. **(f)** Representative traces of $Ca^{2+}$ imaging showing pretreatment with thapsigargin (10 μM, 10 min) blocked the effect of ATP in inducing intracellular $Ca^{2+}$ increase. Scale bar for, 50 μm.

## 3.19 Glutamate regulation of astroglial rapid endocytosis

As close partners to neurons, astrocytes express both ionotropic and metabotropic glutamate receptors (mGluRs) which may be important for neuron-glial interaction (Schools and Kimelberg, 2001). In addition, the application of glutamate can evoke oscillation of intracellular $Ca^{2+}$ in astrocytes via mGluRs (Chen et al., 1997; Fellin et al., 2006). Indeed, in our cultured astrocytes the application of glutamate (1 mM, 5 min) triggered a significant intracellular $Ca^{2+}$ oscillation (Figure 19e). Moreover, the glutamate effect was largely blocked by mGluR antagonist MCPG (200 μM) (Figure 19f) confirming that the intracellular $Ca^{2+}$ oscillation is caused by the activation of mGluR. Since glutamate can be released by both neurons and glial cells, we then examined whether glutamate regulates rapid endocytosis in astrocytes. Before staining astrocytes with FM 1-43 (20 μM) for 3 min, we treat astroglial cells with glutamate (1 mM) for 5 min to induce intracellular $Ca^{2+}$ oscillation. Strikingly, the FM fluorescent signal was remarkably enhanced in glutamate-treated cells in comparison to the control group (Figure 19a-b). Pretreatment of astrocytes with MCPG essentially abolished the effect of glutamate on the rapid endocytosis (Figure 19c-d). These results further indicate that the rapid endocytosis in astrocytes is tightly coupled to the intracellular $Ca^{2+}$ signaling. In addition, these data also suggested that neuro/gliotransmitters such as ATP and glutamate play important roles in regulating the rapid endocytosis because of their effects on intracellular $Ca^{2+}$ in astrocytes.

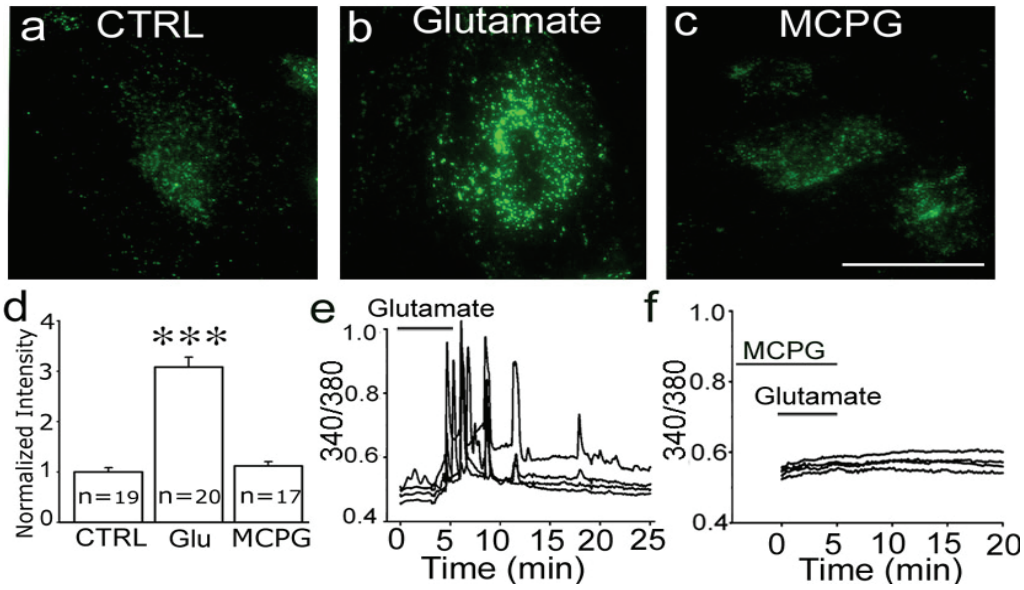


**Figure 19. Rapid endocytosis in astrocytes is enhanced by glutamate stimulation.**

**(a and b)** FM images of control cells **(a)** stained with FM 1-43 (20 μM, 3 min) or cells pretreated with glutamate (1 mM, 5 min; **b**) before conducting the same type of FM staining. Glutamate stimulation greatly enhanced the uptake of FM dye. **(c)** FM image of cells pretreated with mGluR antagonist MCPG (200 μM) before glutamate stimulation and FM staining. **(d)** Normalized intensity of FM signal under control, glutamate, and glutamate + MCPG conditions. FM intensity significantly increased in glutamate treatment group (3.1 ± 0.2, $p<0.001$, Student's *t* test), which was blocked by pretreatment with MCPG (1.1 ± 0.1, $p>0.3$). **(e)** Representative traces showing transient increase of intracellular $Ca^{2+}$ in astrocytes induced by glutamate application (1 mM). **(f)** Representative traces showing that pretreatment with MCPG (200 μM) blocked the $Ca^{2+}$ increase after glutamate stimulation. Scale bar for, 50 μm.

## 3.20 Amyloid β peptide regulates astroglial rapid endocytosis

Alzheimer's disease is characterized by the accumulation of amyloid beta peptide (Aβ) in senile plaques. It has been well established that astrocytes are actively involved in the development of Alzheimer's disease (Nagele et al., 2004). Among different Aβ variants, Aβ 1-42 is believed to be the major toxic component in Aβ plaques, and Aβ 25-35 is the shortest fragment that mimics the effect of full-length Aβ 1-42 (Zameer et al., 2006). In addition, both of these two forms of Aβ have been shown to be capable of inducing robust intracellular $Ca^{2+}$ transients in cultured astrocytes (Abramov et al., 2003). Therefore, we hypothesize that Aβ may affect the rapid endocytosis in astrocytes. To test this possibility, we first examined whether Aβ triggers intracellular $Ca^{2+}$ transients in our cultured astrocytes. Indeed, either Aβ 25-35 or Aβ 1-42 treatment showed robust transients of intracellular $Ca^{2+}$ as shown in $Ca^{2+}$ ratio imaging (Figure 20e & j). For both Aβ peptides, the intracellular $Ca^{2+}$ transients took place after a delay of ~5-15 min which is consistent with previous research (Abramov et al., 2003). We then examined the direct effect of Aβ on the FM-labeled rapid endocytosis in astrocytes. To do that, we treated astrocytes with either Aβ 25-35 or Aβ 1-42 for 15 min first and then stained them with FM 1-43 for 3 min. For control experiments, cells were treated with reverse Aβ peptides or with normal bath solution. As we expected, no difference was found in the FM dye uptake between astrocytes treated with reverse Aβ or normal bath solution (Fig 20a-b and f-g). In contrast, the treatment of

Aβ 25-35 or Aβ 1-42 significantly enhanced FM dye uptake compared to those in control groups (Figure 20 c-d and Fig 20 h-i). These data indicate that the novel endocytic pathway labeled by FM dye in astrocytes may be regulated by pathological agents such as Aβ peptide which affects the intracelluar $Ca^{2+}$.

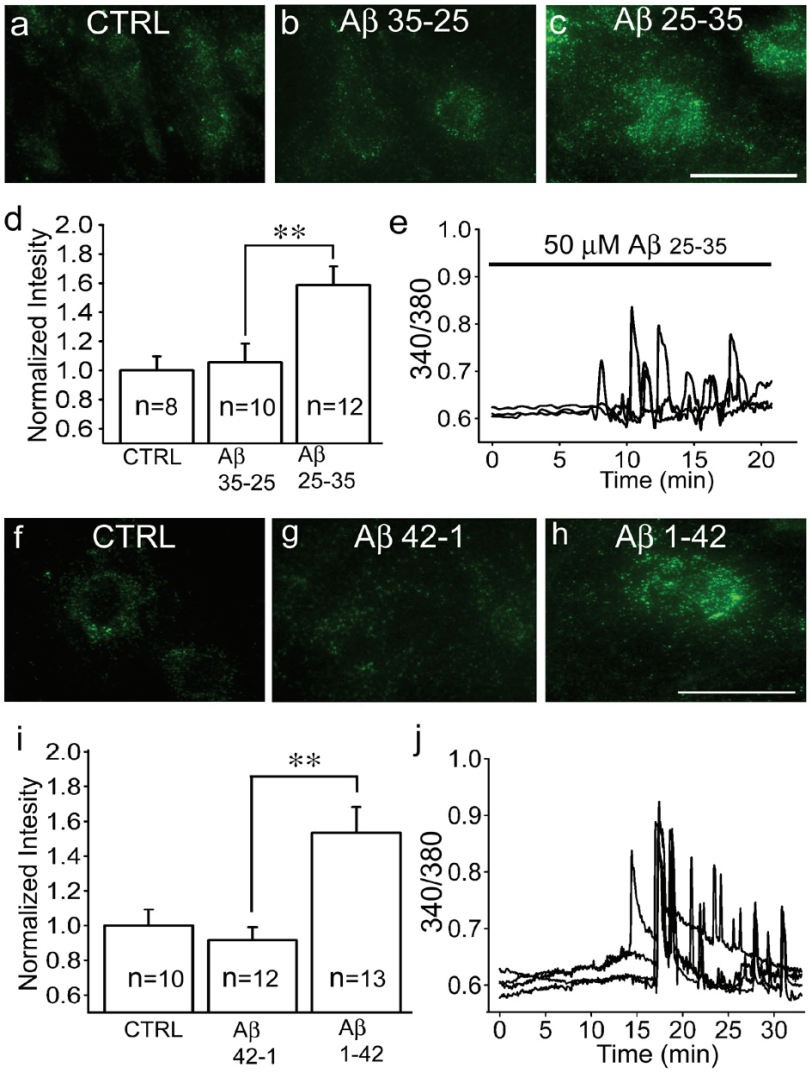


**Figure 20. Rapid endocytosis in astrocytes is upregulated by amyloid β peptide.**

**(a to c)** FM images of astrocytes under control **(a)**, pretreatment with reverse peptide Aβ 35-25 (50 μM, 10 min; **b**), or active peptide Aβ 25-35 (50 μM, 10 min; c). For all 3 groups, astrocytes were stained with FM 1-43 (20 μM) for 3 min. **(d)** Normalized intensity of FM signal in different treatment groups. FM intensity significantly increased in Aβ 25-35 treatment group (1.5 ± 0.1) when compared to the reverse peptide Aβ 35-25 (1.1 ± 1.1, $p<0.009$, Student'*s t* test) or control group ($p<0.002$). **(e)** Representative recordings showing that application of Aβ 25-35 (50 μM) induced significant increase of intracellular $Ca^{2+}$ in astrocytes. **(f to h)** FM images of astrocytes under control (f), pretreatment with reverse peptide Aβ 42-1 (50 μM, 20 min; g), or active long peptide Aβ 1-42 (50 μM, 20 min; h). Scale bar for A – C and F – H, 50 μm. **(i)** Normalized intensity of FM signal in different treatments. FM intensity significantly increased in Aβ 1-42 treatment group (1.5 ± 0.1) compared to the mock treatment with Aβ 42-1 (0.92 ± 0.08, $p<0.002$, Student'*s t* test) or control group ($p<0.007$). (j) Representative traces showing intracellular $Ca^{2+}$ increase in astrocytes induced by application of Aβ 1-42 (50 μM). Scale bar for, 50 μm.

# CHAPTER 4 DISCUSSION

## 4.1 Overview of main findings

We have identified a novel endocytic pathway in astrocytes which has unique features: it is a rapid and constitutive pathway but independent of clathrin. In addition, it is also caveolae- and dynamin-independent but regulated by Rab5, a small GTPase. We also examined which endocytic intracellular compartments are involved and found that after being internalized, FM dye quickly passes through early endosomes and accumulates in late endosomes/lysosomes. More interestingly, we found that FM-dye labeled endocytosis is actively regulated by intracellular $Ca^{2+}$, which is a critical bioactive signal regulating astrocyte functions. Both neuro- and gliotransmitters ATP and glutamate can profoundly regulate this rapid endocytosis, suggesting a novel target between neuron-glial interactions. Moreover, amyloid beta peptide also significantly increases rapid endocytosis through increasing intracellular $Ca^{2+}$ in astrocytes. These results suggest that astroglial cells have a unique endocytic pathway that is independent of clathrin or caveolae but can be tightly regulated by intracellular $Ca^{2+}$ in response to microenvironmental stimuli.

We have summarized our experimental data in a model to illustrate the distinct features of rapid endocytosis in astrocytes (Figure 21). Dynamin-independence and Rab5-dependence of rapid endocytosis in astrocytes indicate a unique endocytic pathway that is different from previously reported clathrin-independent pathways (Lamaze et al., 2001; Sabharanjak et al., 2002). Previous work suggests that non-clathrin- and clathrin-dependent endosomes will merge after endocytosis (Naslavsky et

al., 2003; Sharma et al., 2003). However, our experiments demonstrate that following immediate endocytosis, the majority of FM dye-labeled vesicles are largely separated from the transferrin-carrying endosomes (Figure. 5). Therefore, we propose that the rapid endocytic pathway in astrocytes is distinct from the clathrin- and caveolin-dependent pathway (Figure 21). In addition, the FM dye-labeled vesicles are not targeted to the Golgi complex (Figure 3), whereas clathrin- and caveolin-dependent pathways may interact with the Golgi network (Maxfield and McGraw, 2004; Parton and Simons, 2007). This finding implies that signaling molecules internalized through different endocytic pathways may not be mixed in the cytoplasmic vesicles in order to ensure signaling specificity. Following rapid endocytosis, vesicles move along microtubules to the peri-nuclear area to deposit the internalized solutes into late endosomes/lysosomes, either for degradation or for storage. Recent studies suggest that ATP may be stored inside lysosomes and then released through lysosomal fusion with plasma membranes (Jaiswal et al., 2007; Zhang et al., 2007). The overall process of lysosomal exocytosis appears to be slower than the rapid endocytosis reported here. One possible explanation for the different kinetics between the endocytosis and exocytosis of ATP is that before lysosomal exocytosis, ATP needs to be sorted through early and late endosomes and gradually concentrated in lysosomes. An interesting finding of our work is the $Ca^{2+}$-dependent regulation of rapid endocytosis. This $Ca^{2+}$-dependence suggests that the rapid endocytosis is not a passive process but rather an active trafficking route that can respond to a variety of physiological or pathological stimulants. It is worth noting that the lysosomal release of ATP is also increased by the elevation of intracellular $Ca^{2+}$ (Jaiswal et al., 2007; Zhang et al., 2007), suggesting a $Ca^{2+}$-regulated endocytosis-exocytosis cycling

of ATP signaling in astrocytes (Figure 21). We should point out that ATP and FM dye shown in our model are only two representative molecules that are endocytosed through such rapid endocytosis in the astrocytes. Other signaling molecules such as glutamate and Aβ peptides may also undergo the same endocytosis-exocytosis cycling pathway in astrocytes.

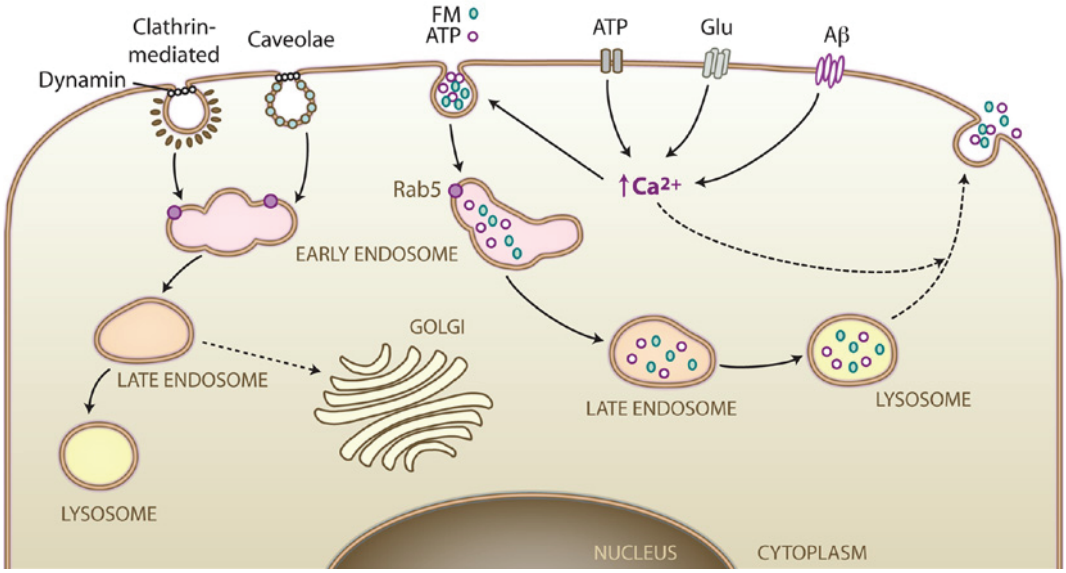


**Figure 21. Model of regulation of fast endocytosis in astrocytes.** The clathrin- and caveolin-dependent pathways are shown on the left. They are dynamin-dependent. The FM- and ATP-labeled endocytic pathway is shown on the right. It is dynamin-independent, and regulated by Rab5 and intracellular $Ca^{2+}$. ATP can undergo an endocytosis-exocytosis cycle through the rapid endocytic pathway. ATP, glutamate, and Aβ peptides are important regulators of the rapid endocytosis in astrocytes.

## 4.2 FM-labeled rapid endocytosis is a novel endocytic pathway

### 4.2.1 FM-labeled endocytosis is independent of clathrin

In mammalian cells, clathrin-mediated endocytosis is considered to be one of the major endocytic entries. It is featured by the formation of clathrin-coated internalized vesicles. Once endocytosed, clathrin-coated vesicles are first decoated and then targeted to early endosomes which are widely recognized as the major sorting organelle in animal cells. It is well established that transferrin and its receptors are typically endocytosed through clathrin-mediated endocytosis. In addition, after being internalized, a significant portion of transferrin is localized within early endosomes before being recycled back to the plasma membrane (Mu et al., 1995; Sheff et al., 2002). In our experiments, we have applied several independent approaches to examine whether clathrin mediates the rapid endocytosis and show that the rapid endocytosis labeled by FM dye in astrocytes is independent of clathrin.

Our conclusions are based on following findings: first, hypertonic solution treatment which has been widely used to disrupt the assembly of clathrin lattice in the plasma membrane does not affect rapid endocytosis in astrocytes (Heuser and Anderson, 1989). Second, the rate of FM dye labeled endocytosis is much faster than clathrin mediated endocytosis. Because of very brief exposure, like 90 s, to FM dye is sufficient for astrocytes to uptake FM. In contrast, transferrin signal only can be detected after 30 min incubation. Third, sequential staining showed that FM dye and transferrin are internalized and sorted to different cellular compartments. Fourth, FM dye is not colocalized with either EGFP-clathrin or endogenous clathrin labeled by anti-clathrin antibody. However, there is possibility that right after being internalized, FM dye quickly accumulates

in intracellular compartments when EGFP-clathrin or anti-clathrin antibody still label the surface clathrin coated pit. To strengthen our conclusion, a more specific functional knock down, such as RNAi, approach could be added to this experiment. Finally, the uptake of FM dye did not require dynamin, which is required to pinch off the clathrin-coated vesicles and caveolae vesicles at the early stage of endocytosis (Konopka et al., 2006). These results indicate that the FM dye-labeled endocytic pathway is distinctly different from the classical clathrin-mediated endocytosis. For future studies, we need to confirm that the anti-clathrin antibody indeed labeled the clathrin coated vesicles. To do that we can immunostain cells with both anti-clathrin antibody and anti-transferrin receptor antibody, theoretically these two immunofluorescent signals should well colocalized, since transferrin receptors are known to be recycled via clathrin-mediated endocytic pathway.

### 4.2.2 FM-labeled endocytosis is independent of caveolae

In addition to clathrin-dependent endocytic pathway, there are other endocytic pathways such as caveolae-mediated endocytosis, which does not require the clathrin coat on endocytosed vesicles (Johannes and Lamaze, 2002). Caveolae are omega-shaped invaginations that are distributed on the plasma membrane. They are highly immobile and often reside in cholesterol-enriched lipid rafts (Henley et al., 1998; Thomsen et al., 2002). In addition, the assembly of caveolae requires coating with caveolin 1 which is considered to be a caveolar structural protein (Rothberg et al., 1992). Unlike clathrin-mediate endocytosis, caveolae endocytosis is not constitutive but rather stimulated by signaling molecules. For example, when SV40 move into the caveolae they trigger the acceleration of membrane-fission reactions which leads to the

detachment of caveolae from the plasma membrane (Pelkmans et al., 2001; Pelkmans et al., 2002). Caveolae have been identified in astrocytes as reported in previous research (Cameron et al., 1997; Cameron et al., 2002) and in our experiments. The FM-labeled fast and constitutive endocytosis we characterized here is unlikely mediated through caveolar endocytic pathway. First of all, treatment of astrocytes with filipin and MβCD that disrupts the formation of caveolae by depleting the membrane cholesterol (Galvez et al., 2004) had no effect on the rapid endocytosis. Second, after being endocytosed, FM dye-labeled vesicles were found not colocalizing with either EGFP-caveolin1 or endogenous caveolin-1 immunopuncta. Third, disrupting the function of dynamin which regulates the budding of caveolae (Oh et al., 1998) did not abolish the rapid endocytosis of FM dye in astrocytes. These independent lines of evidences strongly support the conclusion that the rapid endocytosis in astrocytes is not mediated by caveolae. For the future studies, experiments needed to be added to confirm that the drug treatment indeed disrupts the formation of caveolae in astrocytes.

### 4.2.3. Dynamin-independent rapid endocytosis

Dynamin plays a critical role in regulating the both clathrin-mediated and caveolae-mediated endocytosis. It is recruited to the neck of internalized vesicles and further restricts the invagination to release the vesicles coated by clathrin or caveolin1 (Mayor and Riezman, 2004). However, endocytic pathways that are independent to dynamin also exist. For example, cells overexpressing mutant dynamin can still take up molecular like CtxB or GPI-APs (Sabharanjak et al., 2002). Based on our findings,

the rapid endocytosis labeled by FM dye is also dynamin-independent because it is largely unaffected by the overexpression of mutant dynamin. It then raises the question of whether there are any other molecules serving similar functions as dynamin during the internalization of vesicles in this dynamin- independent endocytic entry. Indeed, in dynamin-independent endocytosis, the primary carries that bud from the cell surface were found to have longer and relatively wider surface invagination compared to clathrin or caveolae coated vesicles. This indicates that dynamin-independent endocytosis may use different molecular mechanisms to scissor the invagination and free the internalized vesicles (Kirkham et al., 2005).

## 4.3 Rab5 regulation rapid endocytosis

In our experiments we have demonstrated that the endocytosis can be regulated by Rab5. Rab5 is a small GTPase that mediates the vesicle internalization and early endosome biogenesis by regulating homotypic fusion of endosomes. To examine how Rab5 affects the rapid endocytosis in astrocytes, we have used two different kinds of Rab5 mutants, Rab5S34N and Rab5Q79L as well as wild type Rab5 to transfect cells. Rab5S34N is a dominant negative form of Rab5, in which its activity of GTPase is defect (Stenmark et al., 1994). In our experiments, we first examined how overexpression of Rab5S34N affect the uptake of transferrin, which is clathrin mediated. Indeed, the transferrin endocytosis is largely blocked in transfected cells, which is consistent with previous research (Dinneen and Ceresa, 2004). In addition, the adverse activity of this dominant Rab5S34N has been further confirmed by the finding that in cells overexpressing Rab5S34N, the formation of early endosomes is also

disrupted.

More importantly, overexpression of Rab5S34N seems to downregulate the rapid endocytosis labeled by FM dye in astrocytes, as much less FM dye was uptaken in transfected cells. It has been reported recently by Shumin's group that slow endocytosis of FM dye in astrocytes can be largely blocked by dominant negative Rab5. In their preparation, cells were loaded with FM dye for several hours (Zhang et al., 2007). The effect of Rab5S34N can be explained by the fact that Rab5 is shown to be critical to the formation of internalized vesicle. It is reported that Rab5 can mediate the actin remodeling during the internalization of vesicles. In addition, the motility of endocytosed vesicles are also mediated by the activity of Rab5 (Nielsen et al., 1999). When the activity of Rab5 is abolished by the overexpression of its dominant negative form, the assembling of actin could be affected which may prevent the invagination from being curved and dissociate from the plasma membrane.

## 4.4 Other GTPase may regulate the rapid endocytosis

There are data showing that in dynamin independent endocytosis, other small GTPase such as CDC42 or proteins belonging to Arf family are actively involved, especially in the early step of endocytosis (Mayor and Pagano, 2007). The ADP-ribosylation factor (ARF) family of proteins belongs to the Ras superfamily of small GTPase. Among the ARF family, ARF1 has been shown to play an important in regulating dynamin independent endocytosis. It activates the CDC42 indirectly, and the activated CDC42 further modulates the dynamics of actin, which is critical for the budding process of the internalized vesicles. It will be interesting to examine whether ARF1 or CDC42 are involved in the rapid endocytosis described by us in astrocytes.

In addition to ARF1, another of ARF family, ARF6, is also found to regulate the membrane trafficking and participate in the clathrin and caveolae independent endocytosis (Donaldson, 2003). ARF6 is located on the plasma membrane, through its GTP cycle, ARF6 moves between the cell surface and its recycling compartment. However, the internalization of ARF6 positive vesicle occurs on a slower time scale than traditional clathrin-mediated endocytosis (Miu et al., 2001). Thus, ARF6 may not be involved in the regulation of the rapid endocytosis we identified in astrocytes.

## 4.5 Functional regulation of astroglial rapid endocytosis

### 4.5.1 Rapid endocytosis is regulated by intracellular $Ca^{2+}$

It is well established that in neuronal terminals, the synaptic vesicle turnover is tightly regulated by $Ca^{2+}$. The synaptic vesicle exocytosis and neurotransmitter release is dependent on the influx of extracellular $Ca^{2+}$(Murthy and De Camilli, 2003; Wu, 2004). We first examined the role of $Ca^{2+}$ in regulating the rapid endocytosis in astrocytes by staining FM 1-43 in $Ca^{2+}$-free bath solution. Interestingly, without extracellular $Ca^{2+}$ the rapid endocytosis has not been affected. Subsequent experiments with $Ca^{2+}$ ionophore A23187 and $Ca^{2+}$ chelator BAPTA-AM showed a significant effect on the rapid endocytosis. Astrocytes respond to a variety of stimuli including mechanical, electrical, and chemical stimulation with a substantial change in the intracellular $Ca^{2+}$ signaling. We demonstrated that both ATP and glutamate strongly upregulate the rapid endocytosis in astrocytes by evoking intracellular $Ca^{2+}$ transients. When the intracellular is depleted by thapsigargin, the effect is largely abolished.

We found that the rapid endocytosis in astrocytes is tightly regulated by intracellular $Ca^{2+}$, suggesting that this endocytic pathway is closely associated with environmental changes. At resting state, the rapid endocytosis in astrocytes is a constitutive process but the endocytic rate is kept at a stable level, possibly due to a very low concentration of resting intracellular $Ca^{2+}$. There is possibility that the oscillation of intracellular $Ca^{2+}$ also upregulates the clathrin mediated endocytosis and thus some FM dye is uptaken via the accelerated clathrin mediated internalization. However, previous research does not support this idea. When the intracellular $Ca^{2+}$ is depleted by chelator, the internalization of insulin receptors, which is clathrin mediated, is unaffected (Carpentier et al., 1992). Furthermore, increasing intracellular seems to result in an inhibition of internalization of surface bound ligands, such as insulin growth factor and epidermal growth factor (Korc et al., 1984; Mossner et al., 1984). Thus, the increase of intracellular $Ca^{2+}$ more likely directly accelerate the clathrin independent rapid endocytosis labeled by FM dye.

Although we demonstrated that intracellular $Ca^{2+}$ actively mediates the rapid endocytosis in astrocytes, the mechanism underlying still needs to be further examined. The change of intracellular $Ca^{2+}$ is considered as an important second messenger in a wide variety of signaling pathways. For example, it has been also well established that elevation of intracellular $Ca^{2+}$ level promotes translocation of PKC from cytosol to plasma membrane and facilitates their activation (Berridge et al., 2003). Furthermore, recent study shows that the activation of PKC regulates the dynamics of actin which could potentially affect endocytosis (Yao et al., 2006). We hypothesize that the increase of intracellular $Ca^{2+}$ may activate certain signaling pathway, for example, PKC. The downstream factors that need to be identified further accelerate the clathrin and caveolae

independent endocytic pathway and facilitate the uptake of FM dye.

### 4.5.2 Rapid endocytosis is regulated by ATP and glutamate

We have demonstrated that the rapid endocytosis in astrocytes is tightly regulated by ATP and glutamate through the activation of intracellular $Ca^{2+}$ oscillations. Astrocytes are known to express both ionotropic and metabotropic receptors. Metabotropic receptors, such as P2Y and mGluRs which can be activated by ATP and glutamate respectively are coupled to second messenger signaling (Fiacco and McCarthy, 2006). In addition, these metabotropic receptors mediate responses to a neurotransmitters including ATP and glutamate by showing intracellular $Ca^{2+}$ oscillations in astrocytes (Fellin et al., 2006). Application of ATP can induce dynamic increases of intracellular $Ca^{2+}$ in astrocytes by binding with P2Y receptors (Porter and McCarthy, 1995; Guthrie et al., 1999). Furthermore, as the major excitatory neurotransmitter in the brain, glutamate also induces substantial increase of intracellular $Ca^{2+}$ by activating mGluRs in glial cells (Pasti et al., 1995; Perea and Araque, 2005; Piet and Jahr, 2007). In astrocytes, calcium oscillations are shown to play an important role in neuron-glial interactions (Fischer et al., 2006; Haydon and Carmignoto, 2006). Therefore, the rapid endocytosis in astrocytes could function in regulating not only glial activities but also neuron-glial interactions.

## 4.6 Function of rapid endocytosis

Astrocytes function in regulating extracellular glutamate, via glutamate transporters and recycling glutamate via glutamine and intermediates of the tricarboxylic acid cycle. By clearing excess glutamate from the extracellular space, astrocytes protect against excitotoxic glutamate that can cause neuronal cell death. More importantly, astroglial cells in the brain is to uptake nutrients from blood vessels (Prat et al., 2001). Since clathrin-mediated endocytosis is responsible for the internalization of membrane receptors, we hypothesize that many soluble bioactive molecules and nutrients may be internalized by astrocytes through the rapid endocytosis revealed by our experiments. In resting conditions, the rapid and constitutive endocytosis in astrocytes can serve as a steady source for the supply of nutrients. In addition, the uptake of rapid endocytosis could be non-selective, unlike clathrin- or caveolin-dependent endocytosis which selectively internalizes specific proteins or ligands. It is also possible that certain kind of pathogen like virus particles can also be internalized via this endocytic entry, which needs to be further examined. After endocytosis, internalized molecules will be sorted through early endosomes in a Rab5-dependent manner, where cargos may be separated and destined to different cellular compartments. It is also possible that certain kinds of pathogen like virus particles can also be internalized via this endocytic entry, which needs to be further examined.

More importantly, the rapid endocytosis labeled by FM in astrocytes could play an important role in regulating cell signaling in response to environmental stimulation. As we discussed before, ATP and glutamate can be released by both neurons and astrocytes to modulate astroglial and neuronal activities (Haydon and Carmignoto, 2006). In addition, we

demonstrate that both ATP and glutamate stimulation can significantly enhance the rapid endocytosis by triggering intracellular $Ca^{2+}$ oscillation in astrocytes. Besides physiological functions, excessive ATP release is often associated with pathological conditions, such as stress, hypoxia, and inflammation (Bodin and Burnstock, 2001). ATP can be hydrolyzed to adenosine which itself is also a bioactive molecule activating neuronal adenosine receptors and inhibit neuronal activity. Moreover, extracellular ATP released from glial cells can trigger interglia propagation of $Ca^{2+}$ waves coordinating global change of glial activities. Therefore, the rapid endocytosis of ATP may be an efficient way to attenuate the ATP-induced $Ca^{2+}$ signaling helping both glia and neurons balance the stimulation. We propose that a transient increase of the rapid endocytosis in astrocytes can serve as a neural protective mechanism by quickly internalizing ATP and other signaling molecules in order to prevent any unwanted long-term effects.

## 4.7 Pathological implications of the astroglial endocytosis

### 4.7.1 Aβ regulates rapid endocytosis in Alzheimer's disease

Malfunction of astrocytes are involved in many neurological disorders including Alzheimer's disease, ischemia, and stroke (Schubert et al., 2001; Takuma et al., 2004). Alzheimer's disease is characterized by substantial deposits of Aβ peptides forming plaques in the brain (Goedert and Spillantini, 2006; Haass and Selkoe, 2007). Aβ may be cytotoxic to neurons as well as astrocytes. Consistent with previous work (Abramov et al., 2003), our studies found that Aβ peptides evoke intracellular $Ca^{2+}$

oscillations in cultured astrocytes.

More importantly, we demonstrated that Aβ significantly enhances the rapid endocytosis in astrocytes (Figure 20). Different from the transient release of ATP and glutamate through vesicular exocytosis (Montana et al., 2006), Aβ peptides may be gradually accumulating in aging brains. In addition, we should notice that the $Ca^{2+}$ oscillations caused by Aβ could arise from different mechanisms than those triggered by ATP or glutamate. Instead of activating metabotropic receptors and triggering the release of $Ca^{2+}$ from its internal store, Aβ could form ion channels on the plasma membrane, which leads to a continuous and substantial $Ca^{2+}$ inflow from extracellular $Ca^{2+}$. Therefore, long-term exposure to Aβ peptides may result in abnormal $Ca^{2+}$ oscillations and prolonged malfunction of the rapid endocytosis in astrocytes. One consequence of such abnormal endocytosis could be the formation of vacuoles in the astrocytes after Aβ peptide treatment reported recently (Nuutinen et al., 2007). Considering the fundamental roles of astrocytes in regulating brain functions, we hypothesize that abnormal regulation of the rapid endocytosis in astrocytes may contribute to neurological disorders such as Alzheimer's disease.

### 4.7.2 Rapid endocytosis and ischemia

In addition to Alzheimer's disease, the rapid endocytosis may also be affected in neuronal injury like ischemia. Glutamate excitotoxicity is an important cause of neuronal death in ischemia. Astrocytes play a major role in regulating extracellular glutamate, via glutamate transporters and

recycling glutamate via glutamine and intermediates of the tricarboxylic acid cycle. In addition, rapid endocytosis of astrocytes may also be responsible for removing excessive glutamate. The glutamate uptake capacity of astrocytes can be compromised under ischemic condition by the reactive oxygen species released from damaged tissue (Chen et al., 2000)

As the closest partner to neurons, astrocytes are more capable of clearing reactive oxygen elements than neurons. The oxidant scavenging mechanisms in astrocytes may function to support neuronal survival during ischemia. For example, neuronal culture with astrocytes are more resistant to toxicity induce by nitric oxide, hydrogen peroxide, or superoxide than neurons cultured alone (Mennerick et al., 1999; Xu et al., 1999). Interestingly, during the development of ischemia the oxygen and glucose deprivation also trigger the intracellular $Ca^{2+}$ transients which could potentially affect the rapid endocytosis. It is proposed that during the ischemia, the rapid endocytosis could become more active due to the intracellular $Ca^{2+}$ oscillation and astrocytes may uptake excessive reactive oxygen components. However, a prolonged toxic stimulation caused by oxygen and glucose deprivation may eventually disrupt the normal function of glial cells and leads to pathological conditions.

# CHAPTER 5. HIGH EFFICIENCY OF $Ca^{2+}$ PHOSPHATE TRANSFECTION PROTOCOL


## Abstract

This protocol describes a high efficiency $Ca^{2+}$-phosphate transfection method with low cell toxicity. The $Ca^{2+}$-phosphate transfection method is widely used in transfecting neurons due to its low cell toxicity and simplicity in use, but the efficiency is typically low (~1-5%). To solve this problem, we have developed a new $Ca^{2+}$-phosphate transfection protocol that increases the efficiency by 10 fold (up to 60%), while maintaining low cell toxicity. First, it is critical to have gentle mixing of DNA-$Ca^{2+}$ solution with phosphate buffer to form homogenous snow-like precipitate (particle size 1-3 μm). Second, the precipitate should be dissolved using slightly acidic culture medium to reduce cell toxicity. The high efficiency of this new protocol makes it possible to transfect single autaptic neurons as well as mature neurons (15-82 DIV) for gene functional analysis. The total length of the protocol is 2 to 4 hours (45 min to 3 hours incubation time).

# INTRODUCTION

Neuronal cells are among the most difficult cells for DNA transfection because they are very sensitive to microenvironmental changes and die easily after transfection. $Ca^{2+}$-phosphate transfection is one of the mostly used methods for transfecting neurons because of the low toxicity and easiness to use (Xia et al., 1996; Holz et al., 2000; Chen et al., 2003a; Micheva et al., 2003; Passafaro et al., 2003; Goetze et al., 2004). However, comparing to some other transfection methods such as liposome fusion and viral infection, the transfection efficiency of classical $Ca^{2+}$-phosphate method is typically very low at about 1-5 % in average (Xia et al., 1996; Craig, 1998; Washbourne and McAllister, 2002).

The ultimate goal for every gene transfer method is to have high transfection efficiency but low cell toxicity. For $Ca^{2+}$-phosphate method, the critical issue is to increase the transfection efficiency significantly in order to broaden its applications in gene functional analysis. We have made a great effort to improve the transfection efficiency and identified some critical steps which might have limited the efficiency in previous protocols. One important element of $Ca^{2+}$-phosphate transfection is the formation of appropriate size of the precipitate particles. It is believed that DNA/$Ca^{2+}$-phosphate precipitate enters cells through endocytosis and some DNA molecules further get into the nucleus(Craig, 1998). Previous $Ca^{2+}$-phosphate transfection protocols often recommend that the DNA/$Ca^{2+}$ solution be thoroughly mixed with phosphate buffer solution by continuous shaking on a vortexer. However, we found that continuous vortexing often resulted in large and unevenly distributed precipitation. A much milder vortexing is recommended here in order to form smaller precipitate particles which may be easily endocytosed into cells. Another critical improvement of this protocol is the dissolution of DNA-$Ca^{2+}$-phosphate precipitate after incubation. We dissolve the precipitate in transfection medium pre-equilibrated in 10% $CO_2$ incubator. This is because our normal culture medium and transfection medium are in 5% $CO_2$ incubator, and 10% $CO_2$ will make the medium more acidic, which dissolves small

$Ca^{2+}$-phosphate particles. We found that dissolving the precipitate significantly lowered the cell toxicity, and allowed for much longer incubation with the precipitate in order to increase substantially the transfection efficiency. Other important steps include performing transfection in a new culture plate and put back the cells into the original plate containing original culture medium after the completion of transfection. With these new improvements, we are able to increase the $Ca^{2+}$-phosphate transfection efficiency up to 60% in low-density primary neuronal cultures, which have never been achieved before. Moreover, such high efficiency is not associated with any significant increase in cell toxicity because of the dissolution of the precipitate and the use of original medium after transfection. We have recently used this improved $Ca^{2+}$-phosphate transfection method in the study of endophilin function in synaptic vesicle endocytosis(Chen et al., 2003b).

## MATERIALS

**REAGENTS:**

Clontech CalPhos™ Mammalian Transfection Kit (BD Bioscience, Palo Alto, CA), including 2 M $CaCl_2$, sterile $H_2O$, and 2x HBS. The pH of HBS is critical (~7.05 to 7.12).

**REAGENT SETUP**

Neuronal Culture Medium: 500 ml MEM (Invitrogen), 5% FBS (HyClone), 10 ml B-27 supplement (Invitrogen), 100 mg $NaHCO_3$, 20 mM D-glucose, 0.5 mM L-glutamine, and 25 U/ml penicillin–streptomycin (Invitrogen).

Transfection Medium: same as culture medium except that FBS was omitted to reduce neuronal death.

**EQUIPMENT:**

VWR MV1 mini vortexer

5% $CO_2$ incubator

10% $CO_2$ incubator

## PROCEDURE

The main procedures of our protocol are illustrated in a flow chart in Figure 1.

**1** Transfer selected coverslips containing cultured neurons from their original well to a new 24-well plate with 0.5 ml pre-warmed transfection medium, which is similar to the culture medium except that it is serum-free. Return both the original and the new plate to the incubator (5% $CO_2$).

**2** **CRITICAL STEP:** formation of the appropriate size of DNA/$Ca^{2+}$-phosphate precipitate is critical to achieve the high efficiency**.**

Prepare the DNA/$Ca^{2+}$-phosphate precipitate using Clontech CalPhos™ Mammalian Transfection Kit (BD Bioscience, Palo Alto, CA).

A typical composition of the precipitate is illustrated in the table below:

| For each 12 mm round coverslip | | |
|---|---|---|
| Solution A | cDNA | 1 μg |
| | 2 M $CaCl_2$ | 3.1 μl |
| | Sterile $H_2O$ | to adjust the total volume of solution A to 25 l |
| Solution B | 2x HBS | 25 μl |

**3** CRITICAL STEP: One important modification of the procedure is that when mixing solution A (cDNA-$Ca^{2+}$-$H_2O$) with solution B (2x HBS), add about 1/8 volume of solution A at a time into solution B by quickly pipetting several times and gently vortexing for only 2-3 seconds (VWR MV1 mini vortexer, speed at 600 rpm).

**4** Incubate the DNA mixture at room temperature for 15 - 20 min without any further vortexing in order to form fine particles of precipitate.

**5** Add the DNA/$Ca^{2+}$-phosphate suspension solution drop-wise to each coverslip (50 μl/coverslip).

**6** Incubate cells with the precipitate for 45 min to 3 hrs in 5% $CO_2$ incubator at 37º C. Longer incubation time increases the transfection efficiency with minimal effect on cell survival because the precipitate is dissolved subsequently. After 20 to 30 min of incubation, if examined under a microscope, the precipitate should be homogenous and look like a cover of snow all over the field (see Figure 2 b and c).

**7** **CRITICAL STEP:** it is critical to dissolve the precipitate after incubation to reduce the cell toxicity. This is a novel step different from previous protocols.

After incubation, the precipitate should be dissolved by incubating with transfection medium pre-equilibrated in a 10% $CO_2$ incubator for 15 to 20 minutes. The 10% $CO_2$ will make the medium more acidic, and $CaPO_4$ will dissolve in acidic solution.

**8** CRITICAL: after adding the 10% $CO_2$-equilibrated medium, return the transfection plate to the 5% $CO_2$ incubator rather than the 10% $CO_2$ incubator. If neurons normally cultured in 5% $CO_2$ incubator are put into 10% $CO_2$ incubator for 15 to 20 min, many cells will die. Under microscopic examination at the end of 15-20 min incubation, the precipitate should largely disappear.

**9** Transfer the transfected coverslips back to their original wells containing the original culture medium (‘conditioned medium’) and transfected neurons can survive more than a week. The original culture medium will keep the cell toxicity to a minimal level.

**10** Check the next day for protein expression. Note that the exogenous protein expression using our $Ca^{2+}$-phosphate transfection method is very rapid. We observed GFP

expression under epifluorescent microscope (Zeiss Axiovert 100) in cultured neurons as early as 4 hours after the transfection.

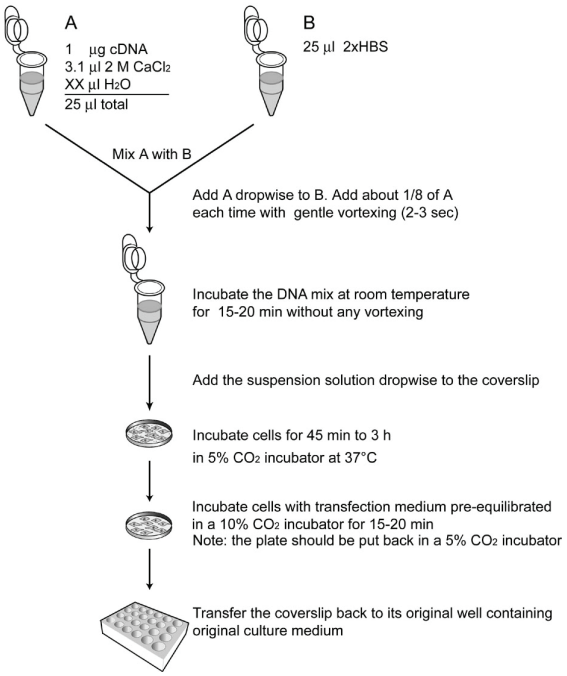


Figure 1 Flow chart of the main procedures of our $Ca^{2+}$-phosphate transfection protocol.

## TIMELINE

| | |
|---|---|
| Step 1 | 3-5 min |
| Step 2 and 3 | 5-10 min |
| Step 4 | 15-25 min |
| Step 5 and 6 | 45 min to 3 hrs |
| Step 7 and 8 | 20-25 min |
| Step 9 | 3-5 min |
| Step 10 | 5-15 min |

## ANTICIPATED RESULTS

### Formation of homogenous DNA-$Ca^{2+}$-phosphate precipitate

We found that continuous vortexing suggested by previous $Ca^{2+}$-phosphate transfection protocols often resulted in large and unevenly distributed precipitation (Figure 2a). We recommend gentle and intermittent vortexing in order to form fine precipitate particles which evenly distributed throughout the whole field like a snow cover (Figure 2b). This homogenous layer of precipitation is critical for high transfection efficiency, possibly because it covers the whole cell surface and the fine precipitate particles are easily endocytosed into cells.

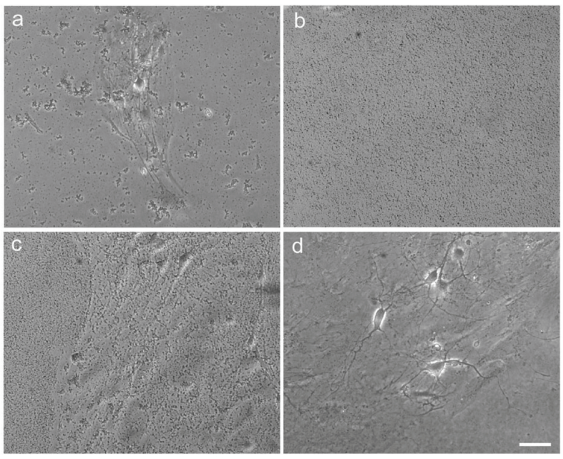


Figure 2 Formation and subsequent dissolution of DNA-$Ca^{2+}$-phosphate precipitate. (a) Continuous vortexing when mixing DNA with $Ca^{2+}$ and phosphate buffer results in large clusters of precipitate (examined after 1 hr incubation). (b, c) Formation of optimal DNA-$Ca^{2+}$-phosphate precipitate through gently vortexing during mixing (image taken after 1 hr incubation). (d) Dissolving precipitate with slightly acidic transfection medium pre-equilibrated in a 10% $CO_2$ incubator. Scale bar, 50 μm. Reproduced from (Jiang et al., 2004).

## Dissolution of the DNA-$Ca^{2+}$-phosphate precipitate

The DNA-$Ca^{2+}$-phosphate precipitate, once formed, will stay with cell cultures. Previous protocols often do not have an effective way to deal with the precipitate. We found that long-term exposure of neurons to the fine precipitation will lead to a high level of cell toxicity. Therefore, it is critical to dissolve the precipitate after transfection. Simple washing with fresh transfection medium could not adequately remove the precipitate, and some neurons might die after repeated washing. We have developed a new method to effectively remove the precipitate using transfection medium pre-equilibrated in a 10% $CO_2$ incubator, which makes the transfection medium slightly more acidic. Importantly, after adding the acidic medium, the plate should be put in the 5% $CO_2$ incubator, not the 10% $CO_2$ incubator because neurons will die if staying there for 15 to 20 min. Figure 2c and 2d illustrate neurons before and after dissolving the DNA-$Ca^{2+}$-phosphate precipitate. The neurons are generally in good health after dissolving the precipitate.

## High transfection efficiency in low-density hippocampal cultures

One advantage of dissolving the DNA-$Ca^{2+}$-phosphate precipitate at any given time is that it allows experimental determination of the optimal incubation time for each individual construct. We found that 45 min to 3 hour incubation time is sufficient for high transfection efficiency while maintaining low cell toxicity. Although 3 hr incubation is longer than what previously used, it does not induce excessive cell death because we dissolve the precipitate after the incubation. Different plasmids may require different incubation time for best transfection. The high transfection efficiency of our improved protocol is best illustrated in Figure 3, where the majority of neurons in a local field are successfully transfected with GFP. For example, in Figure 3c, the entire microisland contained a total of 22 neurons, among which 17 neurons were transfected, corresponding to ~80% transfection efficiency. Quantification of the whole coverslip found 127 transfected neurons out of a total of 211

neurons, yielding a transfection rate of 60.2%, which is far better than an average of 1-5% in previous reports (Xia et al., 1996; Kamiya et al., 1999; Kohrmann et al., 1999). One important note is that using our protocol, the exogenous gene expression is very rapid. We detected GFP fluorescence signal in transfected neurons within 4 hours after transfection. We also imaged GFP-transfected neurons for more than a week after transfection.

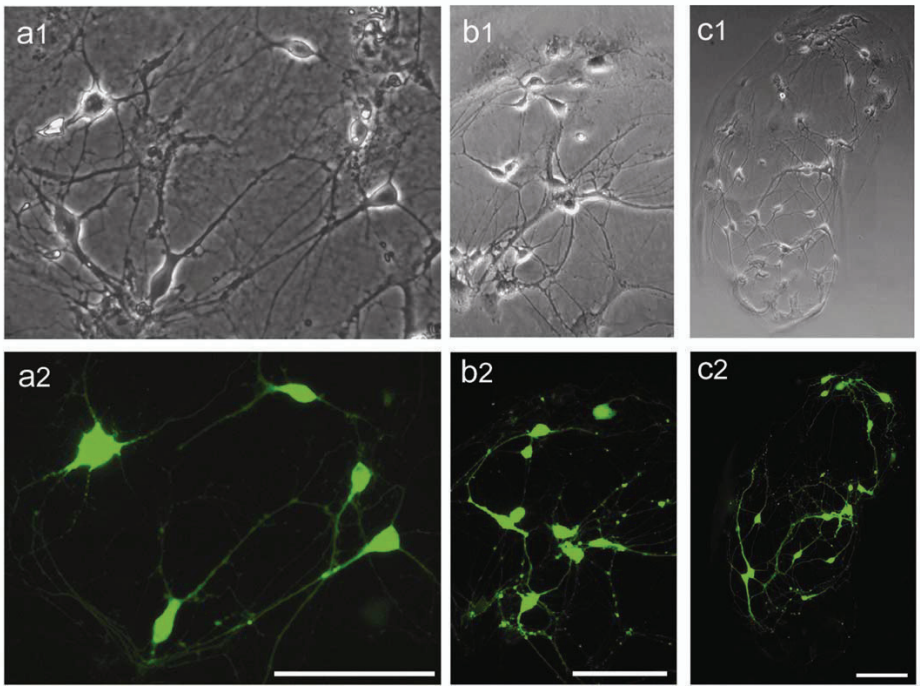


Figure 3 High transfection efficiency achieved with our improved protocol in low-density hippocampal cultures. (a1, b1, c1) Phase-contrast micrographs. (a2, b2, and c2) Fluorescent images of GFP-transfected cells in three independent transfections. Note that the majority of neurons in the local field (microislands) are transfected. Neurons were 10-15 days in culture. Scale bar, 50 μm. Reproduced from (Jiang et al., 2004).

## Transfection of single autaptic neuron in microisland cultures

Microisland cultures containing only a few neurons or even single autaptic neuron have been used to study molecular and cellular mechanisms of synaptic transmission and synaptic plasticity(Bekkers and Stevens, 1991; Chen and van den Pol, 1996; Deng and Chen, 2003; Chen et al., 2004). The dramatic increase of the transfection efficiency of our improved protocol makes it possible to transfect single autaptic neurons in microisland cultures. Figure 4 shows a typical example of a single autaptic neuron transfected with GFP-tagged endophilin. Figure 4a is a phase contrast image and Figure 4b shows the GFP fluorescence image. After transfection, we employed FM fluorescence imaging to study synaptic vesicle cycling (Chen et al., 2003b). We found that FM 4-64 was actively loaded into nerve terminals and then unloaded by depolarizing neurons with a 40 mM $K^+$ solution (Figure 4d).

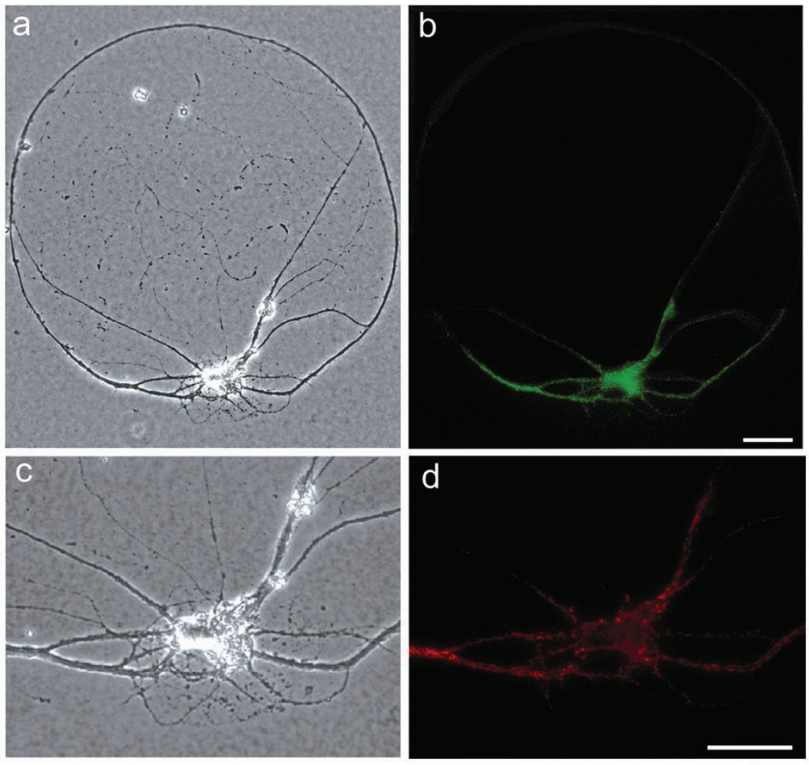


Figure 4 Transfection of single autaptic neurons in microisland cultures. (a) Phase-contrast image of an autaptic neuron in a single microisland. (b) Fluorescent image of the same neuron transfected with GFP-endophilin. (c) High magnification phase-contrast image of

selected field in (a). (d) FM 4-64 labeling of presynaptic terminals (red puncta) demonstrating activity-dependent synaptic vesicle cycling in the transfected neuron. The neuron was 15-day in culture. Scale bar, 50 μm. Reproduced from (Jiang et al., 2004).

## Transfection of mature neurons in culture

The $Ca^{2+}$-phosphate transfection method was often used for transfecting young neurons such as from 2 to 10 days in culture, whereas mature neurons are more difficult to be transfected because they tend to die after transfection. One disadvantage of transfecting young neurons is that the synaptic network is not well established and therefore some gene functional analysis can not be properly carried out. It is ideal for certain experiments that mature neurons can be transiently transfected. Using our improved protocol, we found that neurons at a wide range of ages, from 2 to 82 days in culture, can be successfully transfected (Figure 5 a-d). The successful transfection of 82-day old neurons is remarkable because it is very difficult for neurons to survive for three months in cell culture, yet we are still able to transfect them, suggesting that our protocol has minimal cell toxicity for transfecting mature neurons. More practical application is perhaps the routine transfection of mature neurons around 20 days in culture, when neurons have established substantial synaptic connections (Figure 5C).

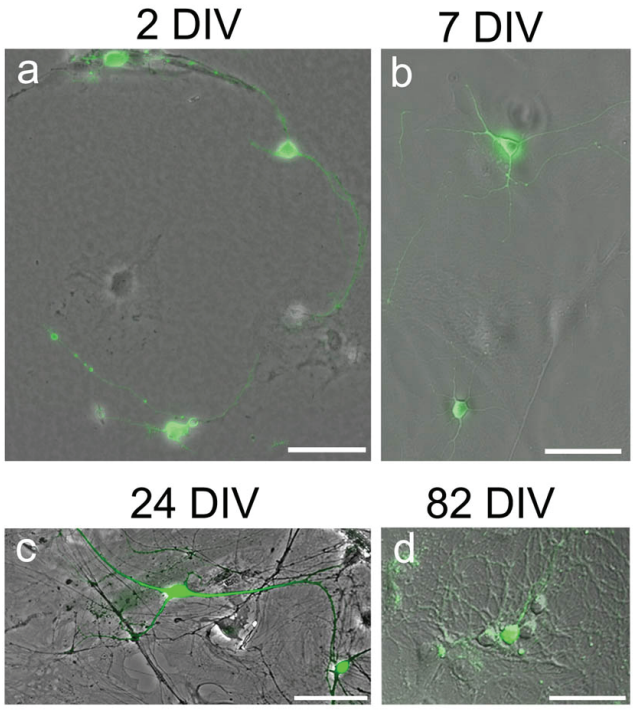


Figure 5 Successful transfection of both mature and immature neurons. (a–b) Transfection of young neurons with EGFP at 2 (a) and 7 (b) days in culture. (c-d) Transfection of mature neurons with EGFP at 24 (c) and 82 (d) days in culture. Scale bar, 50 μm.

## High transfection efficiency in neurons but not glial cells

Our neuronal cultures contain many glial cells, because our neurons are plated on a monolayer of cortical astrocytes. If astrocytes are highly transfected like neurons, it will generate a high background signal (such as in the GFP case) or even directly affect neuronal functions through neuron-glial interaction. Interestingly, although there are numerous glial cells in our cultures, only a few of them are transfected (Figure 6a). One possible explanation is that we used Ara-C to stop glial proliferation, which may also suppress the transfection of glial cells. We also performed transfection in Banker-type culture in which neurons are usually not in contact with glial cells (Figure 6b). The transfection efficacy is similar between low-density cultures with (25.2%) or without

astrocytes (21.6%). In addition, our protocol is applicable to a variety of DNA constructs for transfecting neurons in culture (Figure 6 c-d).

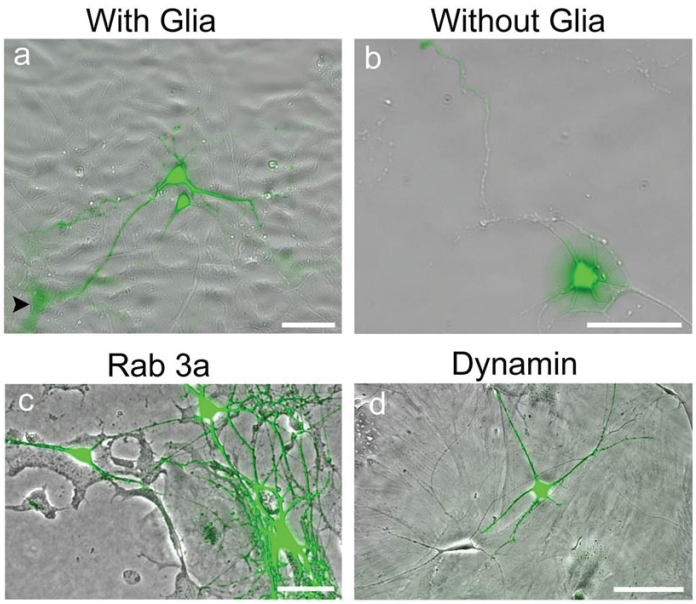


Figure 6 Transfection efficiency in glial cells is very low. (a) Only a few glial cells (arrowhead) are transfected in neuronal cultures with a monolayer of astrocytes. (b) In Banker-type cultures where neurons usually do not contact glial cells directly, transfection efficiency remains high (21.6%). (c-d) Many constructs can be successfully transfected using our protocol. Illustrated here are EGFP-Rab3a (c) and EGFP-dynamin (d). Scale bar, 50 μm.

**TROUBLESHOOTING TABLE**

| Observation | Possible Explanation | Comments and Suggestion |
|---|---|---|
| 1. Poor precipitate formation. | A. The pH of the HBS is not in the optimal range.<br><br>B. The solution A is not mixed properly with solution B. | A. The pH of the HBS should be between 7.05 and 7.12. The pH of the solution may change due to prolonged storage. Always use the HBS solution which is properly stored.<br><br>B. The solution A should be added dropwise into solution B, with 1/8 volume of solution A each time and gentle, intermittent vortexing. Intensive and continuous vortexing may result in large and unevenly distributed particles. No further vortexing is necessary after mixing A and B. |
| 2. Massive cell death after transfection | A. The osmolarity of transfection medium is not the same as that of the culture medium.<br><br>B. Cell toxicity caused by precipitate<br><br>C. Toxicity caused by pH changes<br><br>D. Cell toxicity caused by poor quality of DNA.<br><br>E. Cell toxicity resulting from overexpressed proteins. | A. The osmolarity of the culture medium may increase after long-term cell culture due to constant evaporation in an incubator. The osmolarity of the transfection medium should be adjusted close to that of culture medium (± 3%) by adding sucrose or $H_2O$.<br><br>B. Excessive accumulation of the DNA-$Ca^{2+}$-phosphate precipitate particles inside cells through endocytosis may trigger cell death. Try to shorten the incubation time with the precipitate.<br><br>C. Incubating in 10% $CO_2$ incubator will increase cell death. After adding transfection medium pre-equilibrated in a 10% $CO_2$ incubator, return the plate back in a 5% $CO_2$ incubator.<br><br>D. The cDNA should be prepared with endotoxin free kit. The concentration of cDNA should be ≥ 1 μg/ l.<br><br>E. Overexpression of certain proteins can be stressful to cells. Try to reduce the amount of cDNA and conduct experiments soon after they are expressed. |
| 3. Low transfection efficiency | A. Insufficient precipitate.<br><br>B. Short incubation time.<br><br>C. Inefficient expression due to the profile of the plasmid. | A. See above in 1.A for explanation.<br><br>B. Try to extend the incubation time to get more cells transfected. Usually 1-3 h incubation time is sufficient to ensure high transfection efficiency.<br><br>C. Check whether the cDNA is incorporated correctly into the vector. A well-designed expression vector which includes a strong promoter is helpful to improve the transfection efficiency. |

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# REFERENCES


Abramov AY, Canevari L, Duchen MR (2003) Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity. J Neurosci 23:5088-5095.

Abramov AY, Canevari L, Duchen MR (2004) Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. J Neurosci 24:565-575.

Ahmari SE, Smith SJ (2002) Knowing a nascent synapse when you see it. Neuron 34:333-336.

Ahmari SE, Buchanan J, Smith SJ (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. Nat Neurosci 3:445-451.

Anderson RG (1998) The caveolae membrane system. Annu Rev Biochem 67:199-225.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1998a) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. Eur J Neurosci 10:2129-2142.

Araque A, Sanzgiri RP, Parpura V, Haydon PG (1998b) Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. J Neurosci 18:6822-6829.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208-215.

Araque A, Li N, Doyle RT, Haydon PG (2000) SNARE protein-dependent glutamate release from astrocytes. J Neurosci 20:666-673.

Aravanis AM, Pyle JL, Harata NC, Tsien RW (2003) Imaging single synaptic vesicles undergoing repeated fusion events: kissing, running, and kissing again. Neuropharmacology 45:797-813.

Auld DS, Robitaille R (2003) Perisynaptic Schwann cells at the neuromuscular junction: nerve- and activity-dependent contributions to synaptic efficacy, plasticity, and reinnervation. Neuroscientist 9:144-157.

Auletta M, Nielsen FC, Gammeltoft S (1992) Receptor-mediated endocytosis and degradation of insulin-like growth factor I and II in neonatal rat astrocytes. J Neurosci Res 31:14-20.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFalpha. Science 295:2282-2285.

Behl C, Davis JB, Lesley R, Schubert D (1994) Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77:817-827.

Bekkers JM, Stevens CF (1991) Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell-culture. Proc Natl Acad Sci U S A 88:7834-7838.

Bergles DE, Roberts JD, Somogyi P, Jahr CE (2000) Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. Nature 405:187-191.

Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4:517-529.

Betz WJ, Mao F, Bewick GS (1992) Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. J Neurosci 12:363-375.

Bodin P, Burnstock G (2001) Purinergic signalling: ATP release. Neurochem Res 26:959-969.

Bolte S, Talbot C, Boutte Y, Catrice O, Read ND, Satiat-Jeunemaitre B (2004) FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. J Microsc 214:159-173.

Bonazzi M, Spano S, Turacchio G, Cericola C, Valente C, Colanzi A, Kweon HS, Hsu VW, Polishchuck EV, Polishchuck RS, Sallese M, Pulvirenti T, Corda D, Luini A (2005) CtBP3/BARS drives membrane fission in dynamin-independent transport pathways. Nat Cell Biol 7:570-580.

Bonifacino JS, Glick BS (2004) The mechanisms of vesicle budding and fusion. Cell 116:153-166.

Bowser DN, Khakh BS (2004) ATP excites interneurons and astrocytes to increase synaptic inhibition in neuronal networks. J Neurosci 24:8606-8620.

Brodin L, Low P, Shupliakov O (2000) Sequential steps in clathrin-mediated synaptic vesicle endocytosis. Curr Opin Neurobiol 10:312-320.

Brune T, Deitmer JW (1995) Intracellular acidification and Ca2+ transients in cultured rat cerebellar astrocytes evoked by glutamate agonists and noradrenaline. Glia 14:153-161.

Bucci C, Parton RG, Mather IH, Stunnenberg H, Simons K, Hoflack B, Zerial M (1992) The small GTPase Rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70:715-728.

Burgess TL, Kelly RB (1987) Constitutive and regulated secretion of proteins. Annu Rev Cell Biol 3:243-293.

Cameron PL, Ruffin JW, Bollag R, Rasmussen H, Cameron RS (1997) Identification of caveolin and caveolin-related proteins in the brain. J Neurosci 17:9520-9535.

Cameron PL, Liu C, Smart DK, Hantus ST, Fick JR, Cameron RS (2002) Caveolin-1 expression is maintained in rat and human astroglioma cell lines. Glia 37:275-290.

Carpentier JL, Gorden P, Lew DP (1992) Calcium ions are required for the intracellular routing of insulin and its receptor. Exp Cell Res 198:144-149.

Caselli RJ, Beach TG, Yaari R, Reiman EM (2006) Alzheimer's disease a century later. J Clin Psychiatry 67:1784-1800.

Cavalli V, Corti M, Gruenberg J (2001) Endocytosis and signaling cascades: a close encounter. FEBS Lett 498:190-196.

Ceresa BP, Lotscher M, Schmid SL (2001) Receptor and membrane recycling can occur with unaltered efficiency despite dramatic Rab5(q79l)-induced changes in endosome geometry. J Biol Chem 276:9649-9654.

Chen G, van den Pol AN (1996) Multiple NPY receptors coexist in pre- and postsynaptic sites: inhibition of GABA release in isolated self-innervating SCN neurons. J Neurosci 16:7711-7724.

Chen G, Harata N, Tsien RW (2004) Paired-pulse depression of unitary quantal amplitude at single hippocampal synapses. PNAS 101:1063-1068.

Chen J, Backus KH, Deitmer JW (1997) Intracellular calcium transients and potassium current

oscillations evoked by glutamate in cultured rat astrocytes. J Neurosci 17:7278-7287.

Chen WG, West AE, Tao X, Corfas G, Szentirmay MN, Sawadogo M, Vinson C, Greenberg ME (2003a) Upstream stimulatory factors are mediators of Ca2+-responsive transcription in neurons. J Neurosci 23:2572-2581.

Chen XK, Xiong YF, Zhou Z (2006) "Kiss-and-run" exocytosis in astrocytes. Neuroscientist 12:375-378.

Chen Y, Ying W, Simma V, Copin JC, Chan PH, Swanson RA (2000) Overexpression of Cu,Zn superoxide dismutase attenuates oxidative inhibition of astrocyte glutamate uptake. J Neurochem 75:939-945.

Chen Y, Deng LB, Maeno-Hikichi Y, Lai MZ, Chang SH, Chen G, Zhang JF (2003b) Formation of an endophilin-Ca2+ channel complex is critical for clathrin-mediated synaptic vesicle endocytosis. Cell 115:37-48.

Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, Barnham KJ, Volitakis I, Fraser FW, Kim Y, Huang X, Goldstein LE, Moir RD, Lim JT, Beyreuther K, Zheng H, Tanzi RE, Masters CL, Bush AI (2001) Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. Neuron 30:665-676.

Christoforidis S, Miaczynska M, Ashman K, Wilm M, Zhao L, Yip SC, Waterfield MD, Backer JM, Zerial M (1999) Phosphatidylinositol-3-OH kinases are Rab5 effectors. Nat Cell Biol 1:249-252.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120:421-433.

Cochilla AJ, Angleson JK, Betz WJ (1999) Monitoring secretory membrane with FM1-43 fluorescence. Annu Rev Neurosci 22:1-10.

Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422:37-44.

Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247:470-473.

Craig AM (1998) Transfecting cultured neurons., 2 Edition. Cambridge MA: MIT Press.

Damke H, Baba T, Warnock DE, Schmid SL (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J Cell Biol 127:915-934.

Damm EM, Pelkmans L, Kartenbeck J, Mezzacasa A, Kurzchalia T, Helenius A (2005) Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. J Cell Biol 168:477-488.

Dani JW, Chernjavsky A, Smith SJ (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. Neuron 8:429-440.

Deng LB, Chen G (2003) Cyclothiazide potently inhibits gamma-aminobutyric acid type A receptors in addition to enhancing glutamate responses. PNAS 100:13025-13029.

Dinneen JL, Ceresa BP (2004) Expression of dominant negative Rab5 in HeLa cells regulates endocytic trafficking distal from the plasma membrane. Exp Cell Res 294:509-522.

Domenici MR, Paradisi S, Sacchetti B, Gaudi S, Balduzzi M, Bernardo A, Ajmone-Cat MA, Minghetti L, Malchiodi-Albedi F (2002) The presence of astrocytes enhances beta

amyloid-induced neurotoxicity in hippocampal cell cultures. J Physiol Paris 96:313-316.
Donaldson JG (2003) Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. J Biol Chem 278:41573-41576.
Dringen R, Gutterer JM, Hirrlinger J (2000) Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. Eur J Biochem 267:4912-4916.
Fellin T, Pascual O, Haydon PG (2006) Astrocytes coordinate synaptic networks: balanced excitation and inhibition. Physiology (Bethesda) 21:208-215.
Fiacco TA, McCarthy KD (2004) Intracellular astrocyte calcium waves in situ increase the frequency of spontaneous AMPA receptor currents in CA1 pyramidal neurons. J Neurosci 24:722-732.
Fiacco TA, McCarthy KD (2006) Astrocyte calcium elevations: properties, propagation, and effects on brain signaling. Glia 54:676-690.
Fischer JA, Eun SH, Doolan BT (2006) Endocytosis, endosome trafficking, and the regulation of Drosophila development. Annu Rev Cell Dev Biol 22:181-206.
Friedman HV, Bresler T, Garner CC, Ziv NE (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. Neuron 27:57-69.
Gad H, Low P, Zotova E, Brodin L, Shupliakov O (1998) Dissociation between Ca2+-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron 21:607-616.
Gaiarsa JL, Khalilov I, Gozlan H, Ben-Ari Y (2001) Morphology of CA3 non-pyramidal cells in the developing rat hippocampus. Brain Res Dev Brain Res 127:157-164.
Galvez BG, Matias-Roman S, Yanez-Mo M, Vicente-Manzanares M, Sanchez-Madrid F, Arroyo AG (2004) Caveolae are a novel pathway for membrane-type 1 matrix metalloproteinase traffic in human endothelial cells. Mol Biol Cell 15:678-687.
Ge WP, Yang XJ, Zhang Z, Wang HK, Shen W, Deng QD, Duan S (2006) Long-term potentiation of neuron-glia synapses mediated by Ca2+-permeable AMPA receptors. Science 312:1533-1537.
Glaum SR, Holzwarth JA, Miller RJ (1990) Glutamate receptors activate Ca2+ mobilization and Ca2+ influx into astrocytes. Proc Natl Acad Sci U S A 87:3454-3458.
Glebov OO, Bright NA, Nichols BJ (2006) Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol 8:46-54.
Goedert M, Spillantini MG (2006) A century of Alzheimer's disease. Science 314:777-781.
Goetze B, Grunewald B, Baldassa S, Kiebler M (2004) Chemically controlled formation of a DNA/calcium phosphate coprecipitate: application for transfection of mature hippocampal neurons. J Neurobiol 60:517-525.
Goritz C, Mauch DH, Pfrieger FW (2005) Multiple mechanisms mediate cholesterol-induced synaptogenesis in a CNS neuron. Mol Cell Neurosci 29:190-201.
Goritz C, Mauch DH, Nagler K, Pfrieger FW (2002) Role of glia-derived cholesterol in synaptogenesis: new revelations in the synapse-glia affair. J Physiol Paris 96:257-263.
Granseth B, Odermatt B, Royle SJ, Lagnado L (2006) Clathrin-mediated endocytosis is the

dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron 51:773-786.
Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520-528.
Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 8:101-112.
Hama H, Hara C, Yamaguchi K, Miyawaki A (2004) PKC signaling mediates global enhancement of excitatory synaptogenesis in neurons triggered by local contact with astrocytes. Neuron 41:405-415.
Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2:185-193.
Haydon PG, Carmignoto G (2006) Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev 86:1009-1031.
He L, Wu LG (2007) The debate on the kiss-and-run fusion at synapses. Trends Neurosci 30:447-455.
Henley JR, Krueger EW, Oswald BJ, McNiven MA (1998) Dynamin-mediated internalization of caveolae. J Cell Biol 141:85-99.
Herskovits JS, Burgess CC, Obar RA, Vallee RB (1993) Effects of mutant rat dynamin on endocytosis. J Cell Biol 122:565-578.
Heuser JE, Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. J Cell Biol 108:389-400.
Hinshaw JE (2000) Dynamin and its role in membrane fission. Annu Rev Cell Dev Biol 16:483-519.
Holz RW, Hlubek MD, Sorensen SD, Fisher SK, Balla T, Ozaki S, Prestwich GD, Stuenkel EL, Bittner MA (2000) A pleckstrin homology domain specific for phosphatidylinositol 4, 5-bisphosphate (PtdIns-4,5-P2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P2 as being important in exocytosis. J Biol Chem 275:17878-17885.
Hu R, Cai WQ, Wu XG, Yang Z (2007) Astrocyte-derived estrogen enhances synapse formation and synaptic transmission between cultured neonatal rat cortical neurons. Neuroscience 144:1229-1240.
Jaiswal JK, Fix M, Takano T, Nedergaard M, Simon SM (2007) Resolving vesicle fusion from lysis to monitor calcium-triggered lysosomal exocytosis in astrocytes. Proc Natl Acad Sci U S A 104:14151-14156.
Jiang M, Chen G (2006) High Ca2+-phosphate transfection efficiency in low-density neuronal cultures. Nature Protocols 1:695-700.
Jiang M, Deng L, Chen G (2004) High Ca(2+)-phosphate transfection efficiency enables single neuron gene analysis. Gene Ther 11:1303-1311.
Johannes L, Lamaze C (2002) Clathrin-dependent or not: is it still the question? Traffic 3:443-451.
Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. Nature

396:674-679.

Kamiya Y, Andoh T, Furuya R, Hattori S, Watanabe I, Sasaki T, Ito H, Okumura F (1999) Comparison of the effects of convulsant and depressant barbiturate stereoisomers on AMPA-type glutamate receptors. Anesthesiology 90:1704-1713.

Kawagoe K, Kitamura D, Okabe M, Taniuchi I, Ikawa M, Watanabe T, Kinoshita T, Takeda J (1996) Glycosylphosphatidylinositol-anchor-deficient mice: implications for clonal dominance of mutant cells in paroxysmal nocturnal hemoglobinuria. Blood 87:3600-3606.

Kay AR, Alfonso A, Alford S, Cline HT, Holgado AM, Sakmann B, Snitsarev VA, Stricker TP, Takahashi M, Wu LG (1999) Imaging synaptic activity in intact brain and slices with FM1-43 in C. elegans, lamprey, and rat. Neuron 24:809-817.

Kenworthy AK, Petranova N, Edidin M (2000) High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. Mol Biol Cell 11:1645-1655.

Kirkham M, Fujita A, Chadda R, Nixon SJ, Kurzchalia TV, Sharma DK, Pagano RE, Hancock JF, Mayor S, Parton RG (2005) Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J Cell Biol 168:465-476.

Kish SJ (1997) Brain energy metabolizing enzymes in Alzheimer's disease: alpha-ketoglutarate dehydrogenase complex and cytochrome oxidase. Ann N Y Acad Sci 826:218-228.

Kohrmann M, Haubensak W, Hemraj I, Kaether C, Lessmann VJ, Kiebler MA (1999) Fast, convenient, and effective method to transiently transfect primary hippocampal neurons. J Neurosci Res 58:831-835.

Koizumi S, Fujishita K, Tsuda M, Shigemoto-Mogami Y, Inoue K (2003) Dynamic inhibition of excitatory synaptic transmission by astrocyte-derived ATP in hippocampal cultures. Proc Natl Acad Sci U S A 100:11023-11028.

Konopka CA, Schleede JB, Skop AR, Bednarek SY (2006) Dynamin and cytokinesis. Traffic 7:239-247.

Korc M, Matrisian LM, Magun BE (1984) Cytosolic calcium regulates epidermal growth factor endocytosis in rat pancreas and cultured fibroblasts. Proc Natl Acad Sci U S A 81:461-465.

Kumari S, Mayor S (2008) ARF1 is directly involved in dynamin-independent endocytosis. Nat Cell Biol 10:30-41.

Lakadamyali M, Rust MJ, Zhuang X (2006) Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell 124:997-1009.

Lamaze C, Dujeancourt A, Baba T, Lo CG, Benmerah A, Dautry-Varsat A (2001) Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. Mol Cell 7:661-671.

Lin H, Bhatia R, Lal R (2001) Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology. Faseb J 15:2433-2444.

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci U S A 90:7951-7955.

Luzio JP, Pryor PR, Bright NA (2007) Lysosomes: fusion and function. Nat Rev Mol Cell Biol 8:622-632.

Mackenzie JM, Jones MK, Westaway EG (1999) Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. J Virol 73:9555-9567.

Malchiodi-Albedi F, Domenici MR, Paradisi S, Bernardo A, Ajmone-Cat MA, Minghetti L (2001) Astrocytes contribute to neuronal impairment in beta A toxicity increasing apoptosis in rat hippocampal neurons. Glia 34:68-72.

Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE (1992) beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulneRable to excitotoxicity. J Neurosci 12:376-389.

Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, Pfrieger FW (2001) CNS synaptogenesis promoted by glia-derived cholesterol. Science 294:1354-1357.

Maxfield FR, McGraw TE (2004) Endocytic recycling. Nat Rev Mol Cell Biol 5:121-132.

Mayor S, Riezman H (2004) Sorting GPI-anchored proteins. Nat Rev Mol Cell Biol 5:110-120.

Mayor S, Pagano RE (2007) Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol 8:603-612.

McCarthy KD, de Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85:890-902.

McLauchlan H, Newell J, Morrice N, Osborne A, West M, Smythe E (1998) A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. Curr Biol 8:34-45.

Megias L, Guerri C, Fornas E, Azorin I, Bendala E, Sancho-Tello M, Duran JM, Tomas M, Gomez-Lechon MJ, Renau-Piqueras J (2000) Endocytosis and transcytosis in growing astrocytes in primary culture. Possible implications in neural development. Int J Dev Biol 44:209-221.

Mellman I (1996) Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 12:575-625.

Mellman I, Warren G (2000) The road taken: past and future foundations of membrane traffic. Cell 100:99-112.

Mennerick S, Shen W, Xu W, Benz A, Tanaka K, Shimamoto K, Isenberg KE, Krause JE, Zorumski CF (1999) Substrate turnover by transporters curtails synaptic glutamate transients. J Neurosci 19:9242-9251.

Micheva KD, Buchanan J, Holz RW, Smith SJ (2003) Retrograde regulation of synaptic vesicle endocytosis and recycling. Nat Neurosci 6:925-932.

Miu P, Jarvie KR, Radhakrishnan V, Gates MR, Ogden A, Ornstein PL, Zarrinmayeh H, Ho K, Peters D, GRabell J, Gupta A, Zimmerman DM, Bleakman D (2001) Novel AMPA receptor potentiators LY392098 and LY404187: effects on recombinant human AMPA receptors in vitro. Neuropharmacology 40:976-983.

Montana V, Malarkey EB, Verderio C, Matteoli M, Parpura V (2006) Vesicular transmitter release from astrocytes. Glia 54:700-715.

Morgan C, Colombres M, Nunez MT, Inestrosa NC (2004) Structure and function of amyloid in Alzheimer's disease. Prog Neurobiol 74:323-349.

Mossner J, Logsdon CD, Potau N, Williams JA, Goldfine ID (1984) Effect of intracellular Ca2+ on

insulin-like growth factor II. internalization into pancreatic acini. Roles of insulin and cholecystokinin. J Biol Chem 259:12350-12356.

Mousavi SA, Malerod L, Berg T, Kjeken R (2004) Clathrin-dependent endocytosis. Biochem J 377:1-16.

Mu FT, Callaghan JM, Steele-Mortimer O, Stenmark H, Parton RG, Campbell PL, McCluskey J, Yeo JP, Tock EP, Toh BH (1995) EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. J Biol Chem 270:13503-13511.

Muhlberg AB, Warnock DE, Schmid SL (1997) Domain structure and intramolecular regulation of dynamin GTPase. Embo J 16:6676-6683.

Murthy VN, De Camilli P (2003) Cell biology of the presynaptic terminal. Annu Rev Neurosci 26:701-728.

Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC, Wegiel J (2004) Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol Aging 25:663-674.

Nagler K, Mauch DH, Pfrieger FW (2001) Glia-derived signals induce synapse formation in neurones of the rat central nervous system. J Physiol 533:665-679.

Naslavsky N, Weigert R, Donaldson JG (2003) Convergence of non-clathrin- and clathrin-derived endosomes involves Arf6 inactivation and changes in phosphoinositides. Mol Biol Cell 14:417-431.

Nichols BJ, Lippincott-Schwartz J (2001) Endocytosis without clathrin coats. Trends Cell Biol 11:406-412.

Nielsen E, Severin F, Backer JM, Hyman AA, Zerial M (1999) Rab5 regulates motility of early endosomes on microtubules. Nat Cell Biol 1:376-382.

Nuutinen T, Huuskonen J, Suuronen T, Ojala J, Miettinen R, Salminen A (2007) Amyloid-beta 1-42 induced endocytosis and clusterin/apoJ protein accumulation in cultured human astrocytes. Neurochem Int 50:540-547.

Oh P, McIntosh DP, Schnitzer JE (1998) Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J Cell Biol 141:101-114.

Orlandi PA, Fishman PH (1998) Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. J Cell Biol 141:905-915.

Palade GE (1955) Studies on the endoplasmic reticulum. II. Simple dispositions in cells in situ. J Biophys Biochem Cytol 1:567-582.

Paradis E, Douillard H, Koutroumanis M, Goodyer C, LeBlanc A (1996) Amyloid beta peptide of Alzheimer's disease downregulates Bcl-2 and upregulates bax expression in human neurons. J Neurosci 16:7533-7539.

Paradisi S, Sacchetti B, Balduzzi M, Gaudi S, Malchiodi-Albedi F (2004) Astrocyte modulation of in vitro beta-amyloid neurotoxicity. Glia 46:252-260.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. Nature 369:744-747.

Parton RG, Simons K (2007) The multiple faces of caveolae. Nat Rev Mol Cell Biol 8:185-194.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic purinergic signaling coordinates synaptic networks. Science 310:113-116.

Passafaro M, Nakagawa T, Sala C, Sheng M (2003) Induction of dendritic spines by an extracellular domain of AMPA receptor subunit GluR2. Nature 424:677-681.

Pasti L, Pozzan T, Carmignoto G (1995) Long-lasting changes of calcium oscillations in astrocytes. A new form of glutamate-mediated plasticity. J Biol Chem 270:15203-15210.

Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci 17:7817-7830.

Pasti L, Zonta M, Pozzan T, Vicini S, Carmignoto G (2001) Cytosolic calcium oscillations in astrocytes may regulate exocytotic release of glutamate. J Neurosci 21:477-484.

Pelkmans L, Helenius A (2002) Endocytosis via caveolae. Traffic 3:311-320.

Pelkmans L, Kartenbeck J, Helenius A (2001) Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol 3:473-483.

Pelkmans L, Puntener D, Helenius A (2002) Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. Science 296:535-539.

Pelkmans L, Burli T, Zerial M, Helenius A (2004) Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. Cell 118:767-780.

Perea G, Araque A (2005) Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. J Neurosci 25:2192-2203.

Pfrieger FW (2003) Role of cholesterol in synapse formation and function. Biochim Biophys Acta 1610:271-280.

Pfrieger FW, Barres BA (1997) Synaptic efficacy enhanced by glial cells in vitro. Science 277:1684-1687.

Piet R, Jahr CE (2007) Glutamatergic and purinergic receptor-mediated calcium transients in Bergmann glial cells. J Neurosci 27:4027-4035.

Porter JT, McCarthy KD (1995) Adenosine receptors modulate [Ca2+]i in hippocampal astrocytes in situ. J Neurochem 65:1515-1523.

Porter JT, McCarthy KD (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci 16:5073-5081.

Prat A, Biernacki K, Wosik K, Antel JP (2001) Glial cell influence on the human blood-brain barrier. Glia 36:145-155.

Regland B, Lehmann W, Abedini I, Blennow K, Jonsson M, Karlsson I, Sjogren M, Wallin A, Xilinas M, Gottfries CG (2001) Treatment of Alzheimer's disease with clioquinol. Dement Geriatr Cogn Disord 12:408-414.

Ringstad N, Gad H, Low P, Di Paolo G, Brodin L, Shupliakov O, De Camilli P (1999) Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. Neuron 24:143-154.

Rink J, Ghigo E, Kalaidzidis Y, Zerial M (2005) Rab conversion as a mechanism of progression from early to late endosomes. Cell 122:735-749.

Roberts RL, Barbieri MA, Pryse KM, Chua M, Morisaki JH, Stahl PD (1999) Endosome fusion in living cells overexpressing GFP-Rab5. J Cell Sci 112 ( Pt 21):3667-3675.

Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG (1992) Caveolin, a protein component of caveolae membrane coats. Cell 68:673-682.

Sabharanjak S, Sharma P, Parton RG, Mayor S (2002) GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev Cell 2:411-423.

Schools GP, Kimelberg HK (2001) Metabotropic glutamate receptors in freshly isolated astrocytes from rat hippocampus. Prog Brain Res 132:301-312.

Schubert P, Ogata T, Marchini C, Ferroni S (2001) Glia-related pathomechanisms in Alzheimer's disease: a therapeutic target? Mech Ageing Dev 123:47-57.

Schuske KR, Richmond JE, Matthies DS, Davis WS, Runz S, Rube DA, van der Bliek AM, Jorgensen EM (2003) Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin. Neuron 40:749-762.

Sekiguchi JM, Gao Y, Gu Y, Frank K, Sun Y, Chaudhuri J, Zhu C, Cheng HL, Manis J, Ferguson D, Davidson L, Greenberg ME, Alt FW (1999) Nonhomologous end-joining proteins are required for V(D)J recombination, normal growth, and neurogenesis. Cold Spring Harb Symp Quant Biol 64:169-181.

Self TJ, Oakley SM, Hill SJ (2005) Clathrin-independent internalization of the human histamine H1-receptor in CHO-K1 cells. Br J Pharmacol 146:612-624.

Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81:741-766.

Sharma DK, Choudhury A, Singh RD, Wheatley CL, Marks DL, Pagano RE (2003) Glycosphingolipids internalized via caveolar-related endocytosis rapidly merge with the clathrin pathway in early endosomes and form microdomains for recycling. J Biol Chem 278:7564-7572.

Sheff D, Pelletier L, O'Connell CB, Warren G, Mellman I (2002) Transferrin receptor recycling in the absence of perinuclear recycling endosomes. J Cell Biol 156:797-804.

Silva RF, Mata LM, Gulbenkian S, Brites D (2001) Endocytosis in rat cultured astrocytes is inhibited by unconjugated bilirubin. Neurochem Res 26:793-800.

Simpson JC, Smith DC, Roberts LM, Lord JM (1998) Expression of mutant dynamin protects cells against diphtheria toxin but not against ricin. Exp Cell Res 239:293-300.

Smart EJ, Ying Y, Donzell WC, Anderson RG (1996) A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane. J Biol Chem 271:29427-29435.

Solis GP, Hoegg M, Munderloh C, Schrock Y, Malaga-Trillo E, Rivera-Milla E, Stuermer CA (2007) Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. Biochem J 403:313-322.

Song H, Stevens CF, Gage FH (2002a) Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39-44.

Song HJ, Stevens CF, Gage FH (2002b) Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nat Neurosci 5:438-445.

Sorkin A, Von Zastrow M (2002) Signal transduction and endocytosis: close encounters of many kinds. Nat Rev Mol Cell Biol 3:600-614.

Stenmark H, Parton RG, Steele-Mortimer O, Lutcke A, Gruenberg J, Zerial M (1994) Inhibition of Rab5 GTPase activity stimulates membrane fusion in endocytosis. Embo J 13:1287-1296.

Suadicani SO, Brosnan CF, Scemes E (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J Neurosci 26:1378-1385.

Takuma K, Baba A, Matsuda T (2004) Astrocyte apoptosis: implications for neuroprotection. Prog Neurobiol 72:111-127.

Tanzi RE, Kovacs DM, Kim TW, Moir RD, Guenette SY, Wasco W (1996) The gene defects responsible for familial Alzheimer's disease. Neurobiol Dis 3:159-168.

Theodosis DT (1979) Endocytosis in glial cells (pituicytes) of the rat neurohypophysis demonstrated by incorporation of horseradish peroxidase. Neuroscience 4:417-425.

Thomsen P, Roepstorff K, Stahlhut M, van Deurs B (2002) Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. Mol Biol Cell 13:238-250.

Ullian EM, Sapperstein SK, Christopherson KS, Barres BA (2001) Control of synapse number by glia. Science 291:657-661.

van der Bliek AM, Redelmeier TE, Damke H, Tisdale EJ, Meyerowitz EM, Schmid SL (1993) Mutations in human dynamin block an intermediate stage in coated vesicle formation. J Cell Biol 122:553-563.

Vasudevan C, Han W, Tan Y, Nie Y, Li D, Shome K, Watkins SC, Levitan ES, Romero G (1998) The distribution and translocation of the G protein ADP-ribosylation factor 1 in live cells is determined by its GTPase activity. J Cell Sci 111 ( Pt 9):1277-1285.

Ventura R, Harris KM (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. J Neurosci 19:6897-6906.

Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 6:626-640.

Warnock DE, Baba T, Schmid SL (1997) Ubiquitously expressed dynamin-II has a higher intrinsic GTPase activity and a greater propensity for self-assembly than neuronal dynamin-I. Mol Biol Cell 8:2553-2562.

Washbourne P, McAllister AK (2002) Techniques for gene transfer into neurons. Curr Opin Neurobiol 12:566-573.

Watt JA, Pike CJ, Walencewicz-Wasserman AJ, Cotman CW (1994) Ultrastructural analysis of beta-amyloid-induced apoptosis in cultured hippocampal neurons. Brain Res 661:147-156.

Wu LG (2004) Kinetic regulation of vesicle endocytosis at synapses. Trends Neurosci 27:548-554.

Xia Z, Dudek H, Miranti CK, Greenberg ME (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci 16:5425-5436.

Xu TL, Li JS, Akaike N (1999) Functional properties of ionotropic glutamate receptor channels in rat sacral dorsal commissural neurons. Neuropharmacology 38:659-670.

Yamada E (1955) The fine structure of the renal glomerulus of the mouse. J Histochem

Cytochem 3:309.

Yang N, Huang Y, Jiang J, Frank SJ (2004) Caveolar and lipid raft localization of the growth hormone receptor and its signaling elements: impact on growth hormone signaling. J Biol Chem 279:20898-20905.

Yankner BA, Duffy LK, Kirschner DA (1990) Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. Science 250:279-282.

Yao J, Qi J, Chen G (2006) Actin-dependent activation of presynaptic silent synapses contributes to long-term synaptic plasticity in developing hippocampal neurons. J Neurosci 26:8137-8147.

Zameer A, Schulz P, Wang MS, Sierks MR (2006) Single chain Fv antibodies against the 25-35 Abeta fragment inhibit aggregation and toxicity of Abeta42. Biochemistry 45:11532-11539.

Zerial M, McBride H (2001) Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2:107-117.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971-982.

Zhang Q, Pangrsic T, Kreft M, Krzan M, Li N, Sul JY, Halassa M, Van Bockstaele E, Zorec R, Haydon PG (2004) Fusion-related release of glutamate from astrocytes. J Biol Chem 279:12724-12733.

Zhang Z, Chen G, Zhou W, Song A, Xu T, Luo Q, Wang W, Gu XS, Duan S (2007) Regulated ATP release from astrocytes through lysosome exocytosis. Nat Cell Biol 9:945-953.

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**Jiang M**, Chen G. High $Ca^{2+}$-phosphate transfection efficiency in low-density neuronal cultures. Nature Protocol. 2006, 1:695-700

Yu W, **Jiang M**, Li RW, Miralles CP, Chen G and De Blas AL. Gephyrin clustering is required for the assembly and stability of GABAergic synapses. Molecular And Cellular Neuroscience.2007, 36:484-500.

Qi J, Wang Y, **Jiang M**, Warren P, Chen G. Cyclothiazide induces robust epileptiform activity in rat hippocampal neurons both *In Vitro* and *In Vivo*. Journal of Physiology. 2006, 571:605-18.

**Jiang M,** Deng L, Chen G. High $Ca^{2+}$-phosphate transfection efficiency enables single neuron gene analysis. Gene Therapy. 2004, 11:1303-11.

## Selected Presentations

2005 Neuroscience Meeting (Washington D.C) “Characterization of rapid endocytosis in astrocytes”.