THE EXPRESSION AND TRAFFICKING OF THE METABOTROPIC GLUTAMATE RECEPTOR mGluR1a IN CULTURED HIPPOCAMPAL NEURONS

by

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CERTIFICATE OF APPROVAL

This is certify that the Ph.D. dissertation of

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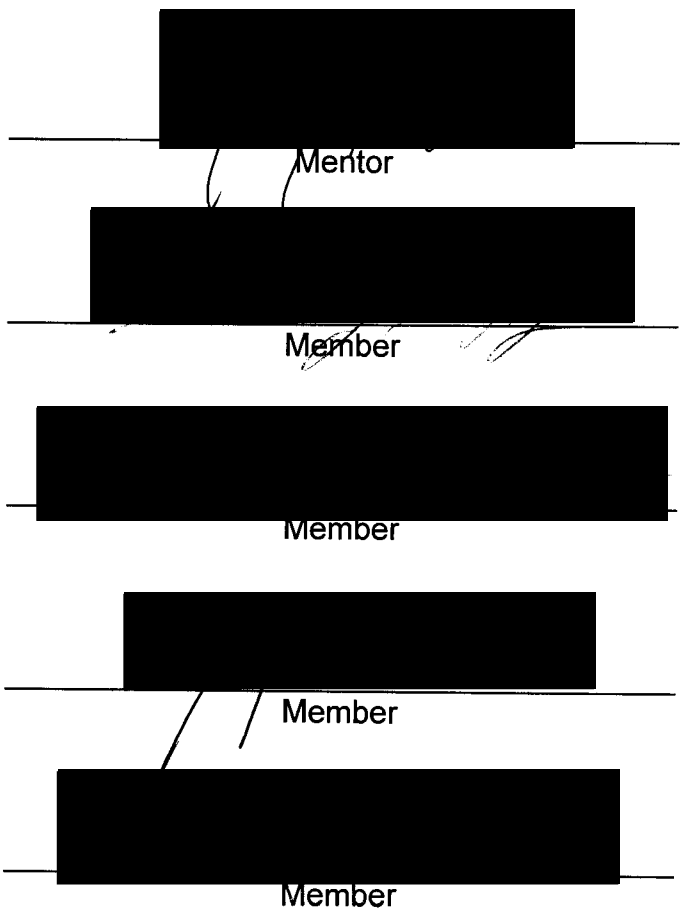
has been approved



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ABSTRACT

Efficient neurotransmission relies on the proper localization of molecular constituents to their appropriate subdomains within neurons. In order to better understand the mechanisms utilized to achieve this goal, we studied the targeting of a protein known to play an important role in neuronal communication -- the metabotropic glutamate receptor, mGluR1a. We first characterized the expression of this receptor in dissociated hippocampal neurons in culture. Next, we expressed a GFP-tagged mGluR1a and confirmed that the protein was correctly localized to the dendritic domain. In order to examine the motifs relevant to this localization, we disrupted select sequences that have been shown previously to mediate an interaction between mGluR1a and either Homer or Tamalin and Shank. These protein-protein interactions have been suggested to mediate mGluR1a trafficking.

In cultured hippocampal neurons, only a subpopulation of interneurons expressed mGluR1a endogenously. mGluR1a-expressing cells fell into two distinct types with very different morphologies and patterns of expression. Type I mGluR1a-expressing neurons possessed longer dendrites that branched far from the soma. Type II mGluR1a-expressing cells had shorter dendrites that branched closer to the cell body. In Type II cells the receptor was localized in distinct "patches" along all dendrites, whereas in Type I cells the receptor exhibited a more uniform pattern. Notably Type II, but not Type I mGluR1a-

expressing cells, co-expressed the peptide somatostatin. In all mGluR1a-expressing cells, the receptor was restricted to dendrites, which were identified based on their expression of MAP2. When GFP-mGluR1a was expressed in cultured neurons, labeled carriers were transported bidirectionally in dendrites with an average velocity of 0.35 μm/second.

When exogenous mGluR1a was expressed in pyramidal neurons, the predominant cell type in hippocampal cultures, the receptor was also present on the dendritic cell surface, where it was synaptically clustered. By mutating and/or deleting select protein interaction motifs, we found that the poly-proline sequence that enables mGluR1a to interact with Homer was necessary for synaptic clustering. In contrast, the PDZ binding domain of mGluR1a, which facilitates its interaction with the proteins Tamalin and Shank, was not necessary for clustering the receptor at synapses. Neither the poly-proline sequence, nor the PDZ binding motif were important for the receptor's dendritic localization. When the entire C-terminus was deleted, the receptor's polarization to dendrites was only slightly reduced, but the same region was sufficient to re-target an unrelated, unpolarized molecule to the dendritic domain.

These results suggest that the dendritic targeting of mGluR1a may be mediated by several, redundant domains. In contrast, synaptic clustering is more discretely regulated by a single motif, indicating that an interaction with the Homer family of proteins via mGluR1a's poly-proline sequence is critical. Taken

together, one can see how multiple regulatory motifs regulate different aspects of receptor trafficking to correctly localize synaptic proteins to appropriate subdomains within the dendritic membrane.

CHAPTER 1

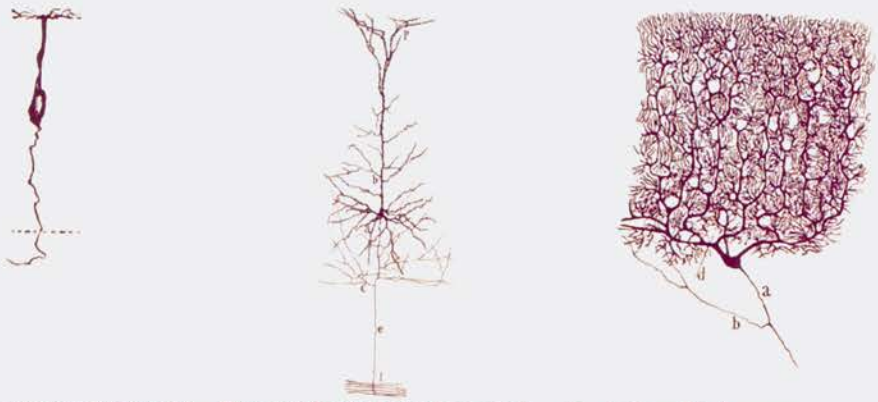
INTRODUCTION

The fundamental role of a neuron is to process and propagate information from one cell to another. This is accomplished through the transformation of an electrical signal to one that is chemical in nature. Thus, efficient communication relies not only on the proper connectivity among correct cell types, but also the precise localization of proteins to specific subcellular compartments within each cell. As neurons have the capacity to form an elaborate architectural framework of processes, the targeting of a protein to specific sites is not a simple undertaking. Rather it depends on the coordinated actions of cellular machinery to ensure that a protein is correctly delivered to its appropriate location.

Following the work of Cajal and others, in 1891 Heinrich Waldeyer formulated the *Neuron Doctrine* — the idea that nerve cells are the fundamental structural units of the nervous system. This novel doctrine found opposition in the *reticularists*, among whom was Camillo Golgi. Reticularists did not believe that nerve cells were independent cellular and functional entities, but rather that they were connected to one another by an elaborate reticular network.

The pioneering anatomical studies of Ramon y Cajal were integral to resolving this debate, showing that neurons were indeed individual entities. Moreover, by refining the staining protocol first developed by Golgi, Cajal also demonstrated through his drawings that neuronal architecture could vary considerably (Figure 1). This morphological diversity, we now know, often indicates the distinct functional roles carried out by individual cell types. Adding another layer of

complexity, studies in more recent years have demonstrated that ultimately, underlying each cell, are molecular differences within each subcellular domain.



Bipolar olfactory receptor neuron in olfactory epithelium.

Pyramidal cell in rabbit cerebral cortex.

Purkinjie cell in human cerebellum.

Figures from Cajal (1909) Histologie du système nerveux de l'homme et des vertébrés. Paris, Maloine.

Figure 1. Examples of neurons with diverse morphologies

"The supreme cunning of the structure of the gray matter is so intricate that it defies and will continue to defy for many centuries the obstinate curiosity of investigators. That apparent disorder of the cerebral jungle, so different from the regularity and symmetry of the spinal cord and of the cerebellum, conceals a profound organization of the utmost subtlety which is at present inaccessible." (from Recollections of My Life by Ramon y Cajal).

Cajal's words are perceptive, for although tools were unavailable at the time, he could distinguish order within the seeming chaos of the "cerebral jungle". The

accessibility of methodologies that now permit researchers to study individual proteins has further defined a level of organization within individual cells.

As a result, many proteins important for neuronal function have been found to possess precise localizations within neurons (for review see Trimmer and Rhodes, 2004). For example, dendritic proteins such as HCN1-2 and Kv2.1 channels are present on distal or proximal dendrites, respectively (Trimmer, 1991; Du, et al 1998; Notomi and Shigemoto, 2004). Ionotropic glutamate receptors are concentrated at the postsynapic density of excitatory synapses (Petralia and Wenthold, 1992; Petralia, et al 1994; Baude, et al 1995; He, et al 1998), whereas GABA receptors exist at inhibitory sites (Somogyi, et al 1989). This precise localization is mirrored on the presynaptic side as well. Studies indicate that neurotransmitter is released at sites in apposition to cognate receptors (Somogyi, et al 1989; Craig, et al 1994). Additionally, the sodium channel $Na_v1.6$ is present at sites where it can best initiate and propagate action potentials – at the axonal initial segment and at nodes of Ranvier (Salzer, 2003). Finally, N ($Ca_v2.2$) and P/Q type ($Ca_v2.1$) calcium channels and much of the protein machinery (including v- and t-SNAREs) important for vesicle docking and fusion are concentrated in presynaptic terminals (Seagar and Takahashi, 1998; Maximov and Bezprozvanny, 2002).

Were each receptor to be indiscriminately distributed on the surface of a neuron, its ability to respond to the release of neurotransmitter from a specific terminal or

to changes in membrane potential would be inefficient. This would in turn have deleterious effects on basic neuronal function, from the activation of downstream signaling events to the propagation of an action potential within a neuron. Ultimately, communication between cells would also be compromised. Such drastic outcomes underline the importance of protein localization in maintaining cellular function.

NEURONAL POLARITY

The proper targeting of proteins is not a characteristic belonging solely to neurons. Rather, the asymmetric distribution of proteins and lipids within the plasma membrane is fundamental to many cells. The segregation of molecular components can be critical to maintaining such cellular functions as vectorial transport, secretion, development, intracellular signaling and cell-cell interactions (Aroeti, et al 1998). In certain instances, domains can be spatially segregated in an otherwise homogenous plasma membrane, as is the case for lipid rafts. These cholesterol and sphingolipid enriched microdomains are present in a lipid environment predominated by phosphatidylcholine and phosphatidylserine (Mayor and Rao, 2004; Meiri, 2004). The compartmentalization of lipids and membrane proteins on the surface of a cell can be important for "containment", thereby limiting downstream signaling events of locally activated receptors (Golub, et al 2004).

In other cases, non-uniform membranes can be present transiently, as in budding yeast (Nelson, 1992) and migrating fibroblasts (Sheetz, 1994 and 1995; see also Figure 2). In each of these cases, there is a rearrangement of the underlying cytoskeleton, and either organelle segregation (yeast) or adhesion molecules (fibroblasts) signaling components in the direction of the bud site or migration, respectively (Sheetz, 1994; Webb et al 2003; Pruyne, et al 2004).

However, this lack of homogeneity is not always limited spatially or temporally across a small span of membrane, but can extend to whole regions of a cell, thereby creating both biochemically and morphologically distinct polarized domains. Two such examples of polarized cells include epithelial cells and neurons. In such cells, which are complex and have whole regions that are physically separate, this segregation of underlying molecular components can prove critical to maintaining the inherent properties of the cell that are important for its function (Figure 2). For epithelial cells, this involves the directional transport of water and solutes from one sector (and therefore

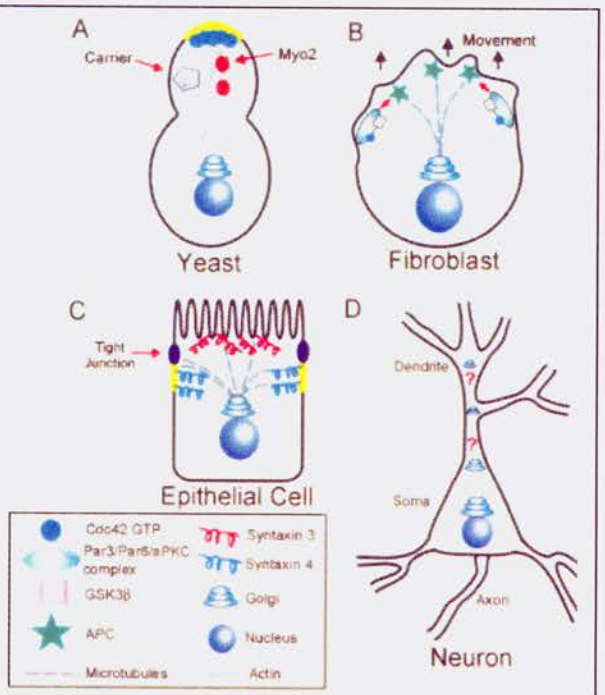


Figure 2. Polarization Is Associated with Rearrangements of the Secretory Apparatus in a Variety of Cell Types. **(A)** In budding yeast, the Rho-GTPase Cdc42 accumulates at sites of membrane addition at the tip of the yeast bud. **(B)** Cdc42-GTP acts in migrating fibroblasts and astrocytes by recruiting the Par3/Par6 complex. **(C)** During epithelial cell polarization, the Golgi apparatus becomes localized to the apical one-third of the cell, and microtubules become rearranged to direct basolateral secretory traffic to the apical two-thirds of the lateral membrane. Selective fusion of cargo with the appropriate domain is accomplished by the polarized distribution of the SNARE proteins syntaxin-3 and syntaxin-4. **(D)** Little is known about how proteins such as Cdc42, the Par complex and the exocyst direct the organelles of the neuronal secretory pathway. *Adapted from Horton and Ehlers, 2003*

membrane domain) to another. There is a similar vectorial flow of information in neurons elicited by changes in ion concentrations that are passed from one compartment to another and ultimately to another cell.

The polarized domains of neurons can be divided into somatodendritic and axonal regions, which differ not only in morphology, but also in their underlying molecular composition and functional roles. Dendrites receive information at synapses where chemical neurotransmitters such as glutamate are released. Hence, receptors must be present on the dendritic membrane to translate ligand binding into an electrical message. Axons, in turn, relay this electrical information to their terminals. As such, those synaptic proteins necessary for subsequent vesicle fusion need to be present presynaptically in order to ensure that an electrical message is again transformed to a chemical one. Thus efficient neuronal communication is dependent on the precise localization of specific proteins at presynaptic terminals and postsynaptic densities.

Because the proper targeting of a protein is a key factor in its eventual cellular function, determining the mechanisms that ensure accurate localization is critical to our understanding how different cellular activities are regulated. Directing proteins to a specific membrane domain is ultimately dependent on the individual steps in trafficking, which include sorting, transport, fusion and endocytosis or retention (Figure 3). The working model of protein trafficking in neurons involves integral membrane protein synthesis in the endoplasmic reticulum (ER), after

which polytopic proteins are passaged through the golgi apparatus. In the golgi, adaptors which recognize specific sequences within the transmembrane proteins may selectively *sort* it away from others proteins. Other signals may be identified directly by transport motors (or other intermediate adaptors), enabling the protein to be *transported* to the plasma membrane. Selective *fusion* of carriers with specific membranes can also play a role in defining the specific localization of a protein. Together, sorting, transport and fusion define steps involved in the initial targeting of a membrane protein, before it has reached the plasma membrane. There are in turn two processes which determine the retention of a protein at the cell surface: the extent to which it is *anchored* there, versus its rate of *endocytosis* -- each of which may rely on additional signals within a polytopic protein. Together, these steps are each integral to the eventual *localization* of all membrane proteins.

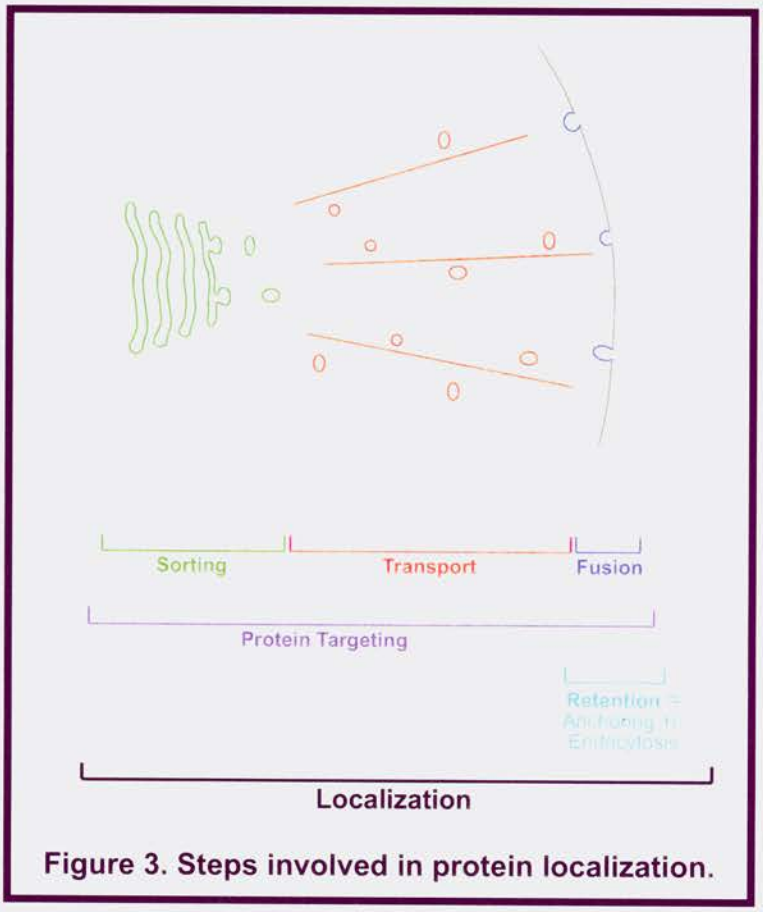


Figure 3. Steps involved in protein localization.

We have learned much about the trafficking of polarized membrane proteins from studies carried out in epithelial cells, specifically MDCK cells, another polarized cell type. These cells have been suggested to possess segregated domains analogous to those in neurons. That is, the basolateral domain has been likened to the somatodendritic cell surface of neurons, whereas the apical domain was thought to be similar to the axonal membrane (Figure 4; Dotti and Simons, 1990). While there are some common mechanisms utilized by both neurons and epithelial cells to segregate membrane proteins to the somatodendritic or basolateral domains (respectively), the notion that *all* sorting mechanisms are common between the two cell types has been increasingly discounted based on studies involving apical proteins which are NOT sorted exclusively to the axonal

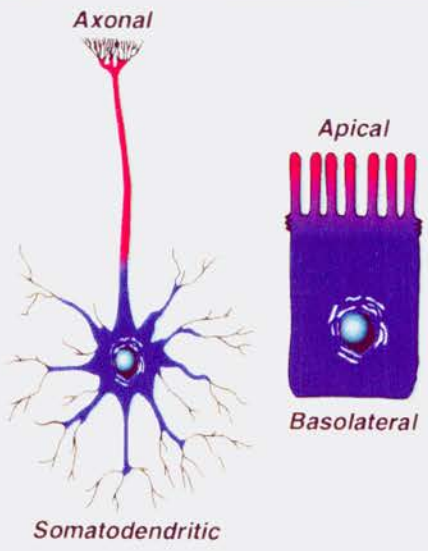


Figure 4. Neurons and epithelia differ in polarized morphology but have a common polarized protein localization system. Neurons are morphologically polarized into axons, which innervate targets, and dendrites, which provide postsynaptic sites to receive input from other neurons. Epithelial cells have apical surfaces which function to exchange nutrients with the lumen. The basolateral surface contains the adhesive cell surface molecules necessary to attach the cell to the basal lamina. Some components of the trafficking system that localizes proteins to the somatodendritic and basolateral compartments (both labeled in blue) seem to be shared by both neurons and epithelial cells. It seems less clear whether the machinery that localizes proteins to axons and apical surfaces (both labeled in red) are shared. *adapted from Rongo, 2001*

domain of neurons (Jareb and Banker, 1998; Cheng et al 2002). Thus, it has become clear that there is not a distinct parallel between apical and axonal targeting signals. Moreover, additional complexity arises when one considers that even some of the basolateral sorting motifs are not receognized as somatodendritic targeting signals (Pietrini, et al 1992; Ghavami, et al 1999; Silverman, et al 2005;). Perhaps neurons have additional or even alternate mechanisms to ensure the proper trafficking of those receptors and ion channels so critical for their ability to communicate with another cell.

As a result of these studies from MDCK cells carried over to neurons, targeting signals necessary for the delivery of membrane proteins to the dendritic domain have been identified. Cytosolic domains that possess short amino acid stretches, often tyrosine based residues, have been found to be important in directing proteins to both the basolateral domain of epithelia and dendritic compartments of neurons, respectively (Craig and Banker, 1994; West, et al 1997; Jareb and Banker, 1998; for review, see Horton and Ehlers, 2003; Winckler and Mellman, 1999).

Thus far, many of the proteins whose targeting has been studied possess a "simple" structure; that is, they are predominantly single pass, transmembrane proteins. Only recently have motifs important for the localization of complex receptors important for neuronal function, such as ionotropic glutamate receptors, been identified. These studies have addressed both the initial steps in

protein trafficking (from the ER to the Golgi) and endocytosis of receptors from the plasma membrane (Bredt and Nicoll, 2003; Perez-Otano and Ehlers, 2004; Vandenberghe and Bredt, 2004; Heusser and Schwappach, 2005; Michelsen, et al 2005). However, few have examined the mechanisms employed by multi-pass transmembrane proteins in their initial targeting to neuronal subdomains (Ghavami, et al 1999; Ruberti and Dotti 2000; Xia, et al 2003). As a result, there is little known regarding how channels and receptor are trafficked to the plasma membrane.

To explore this further, I chose to examine the trafficking of the metabotropic glutamate receptor, mGluR1a, which is also involved in excitatory neurotransmission. Little is known regarding mechanisms used by mGluR1a for its proper trafficking to synapses in neuronal dendrites. This seven transmembrane G-protein coupled receptor is known to homodimerize only with itself, thus posing less of problem to study than those receptors that are capable of oligomerizing with many different subunits. Furthermore, since this receptor is expressed in only a subpopulation of interneurons, expressing the protein in hippocampal neuronal cultures is not a problem. These cultures are composed primarily of pyramidal cells, which do not express mGluR1a, so we need not be concerned that our exogenous receptor may be dimerizing with endogenous mGluR1a.

We have used primary hippocampal neuronal cultures as a model system to study the mechanisms underlying the polarity and clustering of mGluR1a. The advent of this methodology (Banker and Cowan, 1977) has stimulated studies of neuronal cell biology in ways that could not be approached as readily using *in vivo* systems. As a result, it has long been established that dissociated neurons are capable of forming processes with the characteristic features of axons and dendrites in an *in vitro* setting (Banker and Cowan, 1979; Bartlett and Banker, 1984a). The morphological asymetry that develops in these nerve cells is reiterated in a functional polarity that develops in subdomains of each process – presynaptic termini arise from axons while postsynaptic specializations are found in dendrites (Bartlett and Banker, 1984b). Furthermore, the specificity of these connections are such that receptors are present at postsynaptic sites, opposite nerve terminals that release the appropriate neurotransmitter so that ultimately neurotransmission is assured (Craig, et al 1994). Thus this system will be a useful model for investigating the mechanisms underlying mGluR1a trafficking *in vitro* and may likely correspond to those employed *in vivo*.

Using dissociated hippocampal nerve cells, I initially characterized the endogenous distribution of the protein in this system (Chapter 2). I then sought to investigate the role of protein interaction domains present in the receptor and determine how they regulate specific steps in this receptor's localization, namely polarity and clustering. Past studies have focused primarily upon how interacting proteins affect the steady-state localization of mGluR's expressed in

heterologous cells. What has not been addressed is the role that these interactors may have in differentially affecting specific individual steps in protein trafficking in their native system. I thus mutated specific motifs in mGluR1a known to interact with the proteins Homer and Tamalin and evaluated receptor distribution in neurons using the criteria of polarity and clustering (Chapter 3). Before describing these results, I will outline our present understanding of mGluR1a in terms of its trafficking, and the role that other proteins with which it has been linked may play a role in this process.

mGluR1a

G-protein coupled receptors (GPCRs) comprise the largest family of membrane receptors in mammals. They function in response to agonist application by activating downstream signaling cascades mediated by heterotrimeric G proteins. These receptors, which have a characteristic topology that consists of seven transmembrane segments, can be classified into three primary families based on sequence homology: Family A, B and C (Pierce, et al 2002).

Family A, the largest family, encodes receptors that respond to such varied stimuli as light (rhodopsin), adrenaline (adrenergic receptors) and odor (olfactory receptors). Receptors that compose Family B include those for gastrointestinal peptides, calcitonin, parathyroid hormone and corticotropin releasing hormone. Family C includes receptors for metabotropic glutamate (mGluRs), and $GABA_B$, as well as calcium sensing receptors (CaR) and several taste receptors (Pierce, et al 2002).

The family of metabotropic glutamate receptors is composed of 8 different receptors (mGluRs 1-8), whose complexity is further increased by splice variants. Using expression cloning techniques, two groups independently isolated mGluR1a in 1991 (Masu, et al 1991; Houamed, et al 1991). Its sequence was distinct from that of other GPCRs and thus mGluR1a distinguished itself as the

first member of a new family of GPCRs (Family C). mGluR1a encodes a receptor consisting of 1199 amino acids that has a molecular weight of approximately 140 kilodaltons as a monomer.

mGluRs are subdivided into three groups based on signaling pathways, pharmacology and sequence homology as follows: Group I: mGluR1a-e and mGluR5a-b; Group II: mGluR2 and mGluR3; GroupIII: mGluR4, 6, 7, and 8 (Pin and Duvoisin, 1995). Group I mGluRs couple positively to G_q, which activates phospholipase C and leads to the generation of diacylgycerol (DAG) and inositol trisphosphate (IP_3). Protein kinase C (PKC) is in turn, activated by DAG. Ca^{2+} is mobilized from intracellular stores by IP_3 acting at IP_3 receptors (Hermans and Chaliss 2001). In contrast to this, both group II and III mGluRs negatively couple to G_i and thus lead to inhibition of adenylyl cyclase.

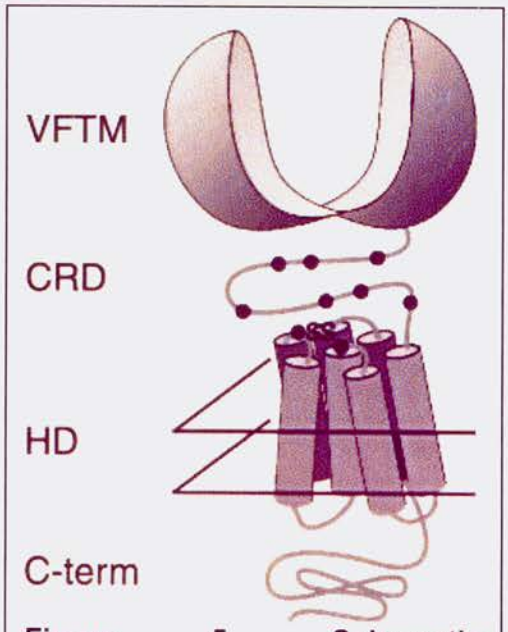


Figure 5. Schematic representation of a family 3 GPCR. These receptors are modular proteins constituted of an N-terminal extracellular venus flytrap module (VFTM) connected to the heptahelical domain (HD) via a cysteine-rich domain (CRD) and terminated by a variable C-terminal intracellular tail containing several binding sites for intracellular proteins. *adapted from Pin, et al 2003*

Group I mGluRs (indeed all members of family C) have very large extracellular

amino and intracellular carboxyl termini (with the exception of shorter splice variants). The N-terminus can be subdivided into two domains: 1) a venus flytrap module which is involved in agonist binding and 2) a cysteine-rich domain that connects the venus flytrap module to the transmembrane segments (Figure 5). The venus flytrap module shares homology with the extracellular domain of ionotropic glutamate receptors as well as bacterial amino acid binding proteins — both of which must first bind agonists before they can function. The cysteine-rich domain is important (although not essential) for dimerization between receptors; however its precise role is not certain. G-protein coupling is achieved via the 2nd intracellular loop of Group I mGluRs. The intracellular C-terminus is variable among family members and is the site of interaction for a number of scaffolding proteins, including Homer, Tamalin and Shank (Brakeman, et al, 1997; Tu, et al 1999; Kitano, et al 2002) and also functions in localizing long forms of mGluRs to postsynaptic domains in neurons (for review see Bockaert and Pin, 1999; Hermans and Challiss, 2001).

While it was once thought that G-protein coupled receptors were functional monomers, studies over the past 10 years show that these proteins function as homo and heterodimeric receptors (Terrillon and Bouvier, 2004; Gomes, et al 2001). Furthermore, this oligomeric structure forms independent of ligand binding (Angers, et al 2002). In general, stringent quality control measures exist to ensure that those oligomeric proteins that have not correctly assembled into the correct quaternary structure are not allowed to progress through the

secretory pathway (Reddy and Corley, 1998). It is no different for GPCRs, which often have consensus amino acid sequence motifs (RKR) to ensure that these receptors are held in the endoplasmic reticulum (ER) until they can dimerize and fold properly. Dimerization may mask ER retention signals in mGluRs, allowing trafficking to the cell surface, as is the case for $GABA_B$ receptors (Margeta-Mitrovic et al 2000; for review, see Michelsen, et al 2005).

In the case of Group I mGluRs, residues in the extracellular amino terminus are responsible for promoting dimerization (Romano, et al 1996 and 2001; Robbins, et al 1999). While there are extracellular cysteine residues that are critical for this process, other domains (including the C-terminus) can facilitate dimerization (Romano, et al 2001; Robbins, et al 1999). Interestingly, heterodimerization does not seem to occur between Group I mGluRs. mGluR1a does not dimerize with mGluR5 (Romano, et al 1996) or even with other mGluR1 splice variants, such as mGluR1b (Robbins, et al 1999). While the N-termini of mGluRs are necessary for dimerization, they may not be the only determinants. Since mGluR1a and mGluR1b differ only in their carboxyl domains, this region must further specify the dimerization interaction. The importance of this finding is that since heterodimerization between members of this same family does not occur, receptor distribution is presumably determined by a homogenous population of receptors.

Many studies have assessed the distribution of mGluR1a in rat brain and have found it to be highly expressed in the olfactory bulb, thalamus, hippocampus and especially the cerebellum (Martin, et al 1992; Baude, et al 1993). In the hippocampus, mGluR1a is not present in pyramidal cells; rather it is localized to a subpopulation of interneurons that are also positive for specific neuropeptides (Baude, et al 1993; Ferraguti, et al 2004). With regard to its subcellular localization, mGluR1a is very precisely located in dendrites in the annulus surrounding postsynaptic densities (Baude, et al 1993; Lopez-Bendito, et al 2001 and 2002). This corresponds to perisynaptic and extrasynaptic regions and this distribution was quantified at cerebellar purkinje cells synapses (Lopez-Bendito, et al 2001). Moreover, this is specific for long forms of Group I mGluRs, as shorter splice variants are not so restricted to synaptic sites (Mateos, et al 2000).

While molecular determinants of mGluR1a dendritic localization have not been elucidated, its clustering (presumably at synaptic sites) is thought to be dependent upon its interaction with the Homer family of proteins (Tu, et al 1998; Tadokoro, et al 1999). Though many of the studies have been carried out in heterologous cells, a role for the Homer interaction motif in anchoring Group I mGluRs in neuronal cells has been demonstrated (Serge, et al 2002; see below as well)

Because mGluR1a is localized to peri/extrasynaptic sites, synaptic activation of mGluRs requires repeated synaptic stimulation, and subsequent spillover of

glutamate to activate the receptor (Coutinho and Knopfel, 2002). Group I mGluRs are also thought to play a role in the molecular changes associated with learning and memory, both long term potentiation (LTP) and long term depression (LTD). Whereas AMPA receptor insertion at synapses is associated with LTP, its removal via endocytosis is thought to underlie LTD. Though LTP deficits measured in CA1 and CA3 are most likely due to mGluR5 or mGluR1 splice variants, and not mGluR1a specifically (which is not expressed in pyramidal neurons; Coutinho and Knopfel), the role of mGluR1a in LTD is more prominent. At the parallel and climbing fiber-purkinje cell synapse, synaptic activation of mGluR1a, and the subsequent rise of intracellular Ca2+ results in PKC activation and the resultant phosphorylation and endocytosis of the AMPA receptor GluR2 (Malenka and Bear, 2004).

A common mechanism employed by many classes of GPCRs to limit downstream signaling in response to prolonged exposure to agonist is desensitization, whereby the presence of agonist does not lead to further activation (von Zastrow, 2001). This can be achieved by phosphorylation of the receptor, which results in a change in its conformation, by receptor endocytosis, or both. Endocytosis is followed by resensitization, which may involve dephosphorylation of the receptor and recycling back to the cell surface. If a receptor is not returned to the plasma membrane, it may undergo down-regulation via degradation, a decrease in synthesis, or both (Seachrist and Ferguson, 2003). mGluR1a internalization has been demonstrated to occur not

only in response to agonist but also in the absence of agonist. Though the former mechanism is arrestin and dynamin-dependent, the latter pathway is not (Dale, et al 2001; but see Pula, et al 2004). While both agonist-dependent and independent internalization of mGluR1a may proceed via clathrin recruitment, other proteins may be employed for each specific pathway (Dale, et al 2002). Thus, certain small GTPases, such as Rab, Ral and ARF, may be called upon in the process of trafficking these GPCRs to and from the plasma membrane and between vesicular (endosomal) compartments (for review, see Bhattarcharya, et al 2004a). More specifically, it has been demonstrated that Ral aids in the constitutive endocytosis of mGluR1a in heterologous cells and possibly to some extent in neurons as well (Bhattarcharya, et al 2004b). Perhaps the internalization of this receptor in response to agonist is the result of coupling to Arf6 and Arf6-GEFs such as Tamalin (see below), as this family of proteins has been implicated in the endocytosis of other GPCRs (Bhattarcharya, et al 2004a; Claing, et al 2001).

TRAFFICKING MOTIFS IN mGluR1a

In order to approach this study of mGluR1a, I first examined the primary structure of this protein for recognizable sequences which may play a role in its trafficking. The two most obvious motifs present in mGluR1a reside in the receptor's carboxyl domain (Figure 6). The first is a poly-proline motif which mediates an interaction between Homer and mGluR1a (Tu, et al 1998). The second is a PDZ binding motif that enables the receptor to bind the proteins tamalin and shank (Tu, et al 1999; Kitano, et al 2002). The role that these specific motifs play in mGluR1a trafficking will be discussed in their respective sections (below). In addition to these, we identified a number of other sequences, including an ER retention and putative ER export motif, an acidic cluster, a tyrosine-based endocytic signal and several di-hydrophobic sequences (Figure 6).

Arginine based sequences (Φ/Ψ/R-R-X-R) have been found in a number of multi-transmembrane proteins, such as potassium channels and glutamate receptors, where they function to retain these multimeric proteins in the endoplasmic reticulum (ER) until they are able to assemble properly (Zerangue, et al 1999; Standley, et al 2000; for review see Michelsen, et al 2005; Vandenberghe and Bredt, 2004). ER export signals have also been defined in channels and receptors. They facilitate exit from the ER, once the correct subunit assembly of receptor complexes has occurred. Another means by which these proteins reach the cell surface is through the involvement of scaffolding proteins, which can help

to traffic such complexes through to the cell surface (Ma and Jan, 2002; Vandenberghe and Bredt, 2004).

mGluR1a has an ER retention sequence (IF**RRKK**) that is slightly different from the consensus sequence but has been demonstrated to be important in regulating the cell surface expression of this receptor (Chan, et al 2001). There is also a putative ER export signal (FVYERE). Although its role in expediting mGluR1a's exit from the ER has not been tested, its presence in the long form of mGluR1 splice variants (mGluR1a, not mGluR1b) could account for the fact that mGluR1b is less efficient at reaching the plasma membrane (Chan, et al 2001).

Motifs in the Cytosolic C-Terminus of mGluR1a

Figure 6. mGluR1a Topology and C-Terminal Motifs. Amino acid sequence of the cytoplasmic tail of mGluR1a, Boxed Regions indicate the following defined and putative motifs: = Homer Interaction Motif; = Tamalin/Shank Interaction Sequence; = 7th transmembrane domain; = ER Retention Motif; = Splice Site; = AcidicCluster/Putative PACS Binding Motif; = Putative ER Export Signal; = Consensus Tyrosine Motif; = Di-Hydrophobic/Endocytic Sequence

Regarding endocytic motifs, a YXXΦ and two dyhydrophobic motifs (one of which is the consensus D/E-X-X-X-L-L) are present in the C-term of mGluR1a. These

sequences have been shown to regulate the sorting of other transmembrane proteins, their endocytosis, or both (Gu, et al 2001). Although studies have been carried out in regards to mGluR1a endocytosis (Dale, et al 2001; Bhattarcharya, et al 2004), no specific residues regulating this process have been identified. The importance of tyrosine residues (Jareb and Banker, 1998) but not di-hydrophobic motifs (Silverman, et al 2005) in polarized targeting of membrane proteins has been addressed.

The acidic cluster sequence in the carboxyl domain of mGluR1a can potentially participate in trafficking as well. Phosphofurin acidic cluster sorting protein (PACS-1) has been shown to interact with furin at acidic cluster domains that are phosphorylated by casein kinase II (CKII) and to regulate its cycling at the golgi and between the plasma membrane and endosomes (Molloy, et al 1999). mGluR1a has two acidic cluster domains with putative CKII phosphorylation sites (S/T-X-X-D/E; see above). Furthermore, mGluR5 has recently been demonstrated to interact with PACS-1 and PACS-2, further suggesting that Group I mGluRs may be regulated by these sorting proteins (Farr, et al, SFN Abstracts 2005).

While each of the aforementioned motifs are likely targets of adaptors and other proteins important in cellular trafficking, the cognate interacting proteins for these specific sequences have yet to be identified. Furthermore, their role in trafficking for mGluR1a is based only on extrapolation from the literature, where they have

been shown to play a role in the localization of other receptors. In this dissertation I focused instead on two protein interaction motifs (see below) that bind to specific proteins that have been suggested to play an active part in mGluR1a localization.

PROTEINS THAT INTERACT WITH mGluR1a

mGluR1a has been found to interact with a number of proteins in recent years, the functional implications of which are still being elucidated. I will highlight those interactions that appear to have the most significance for mGluR1a trafficking thus far. These include the three proteins that have been demonstrated to directly interact with mGluR1a --- Homer, Tamalin and Shank (Tu, et al 1998 and 1999; Kitano, et al 2002).

Homer

Homer was first isolated in 1997 as an immediate early gene induced by electrical activity (Brakeman, et al 1997). This study found that Homer associated with members of the Group I mGluR family of receptors (Brakeman, et al 1997): mGluR1a and mGluR5. The protein identified by Brakeman and colleagues was subsequently termed Homer 1a. The Homer family of proteins is comprised of 3 genes in mammalian species (rat, mouse and human) that are alternatively spliced to generate Homer 1a/b/c, Homer 2a/b and Homer 3, (Xiao, et al 1998; Shiraishi, et al 2004). While the other members of the Homer family are all constitutively expressed in rat brain; Homer 1a, in contrast, is induced in response to activity (Brakeman, et al 1997).

All members of the Homer protein family possess an Ena-VASP Homology 1

domain (EVH1) that interacts with other proteins via a polyproline motif. Some Homer proteins also contain a carboxy-terminal leucine zipper motif that forms a structural coiled-coil domain; Homer 1a lacks the leucine zipper motif (Xiao, et al 1998). While the amino terminus of Homer proteins is responsible for binding to Group I mGluRs, the C-terminal domain mediates association *between* Homer proteins via the coiled-coil region.

The interaction domain in mGluR1a that enables it to bind Homer proteins is in the C-terminus and consists of the following sequence: PPXXFR (Tu, et al 1998). The critical residues mediating this interaction were found to be the initial two proline residues and phenylalanine residue (Tu, et al 1998). In addition to interacting with several Group I mGluRs, Homer also binds other scaffolding molecules such as Shank, which in turn interacts with NMDA receptors via the scaffolding molecules GKAP and PSD-95 (Tu, et al 1999). Additionally, Homer interacts with IP_3 receptors (in the endoplasmic reticulum), and TRP channels (on the cell surface). As such, long forms of Homer can serve as a scaffold (see Figure 7), linking plasma membrane receptors to those localized on intracellular membranes. However, since Homer 1a does not have a C-terminal coiled-coil region, it is thought to act as an endogenous dominant negative during increased synaptic activity, disrupting interactions between Group I mGluR's and constitutively expressed Homer proteins in neurons (Tu, et al. 1998; Xiao, et al. 1998; Yuan, et al 2003; Kim, et al 2003).

Although recent studies have consistently reported that co-expression of mGluR1a (or mGluR5a/b) with Homer 1a increases cell surface expression of the glutamate receptor, the role of the other Homer proteins, especially Homer 1b/c, in directing Group I mGluR's to the surface seems to depend on cell type (Ango, et al. 2002; Ciruela, et al 1999a and 2000; Roche, et al. 1999). Homer 1b is responsible for increased intracellular retention of mGluR1a in HeLa cells whereas Homer 1c (which has an 11 amino acid insertion preceding the coiled-coil domain) leads to increased cell surface expression and clustering of mGluR1a or mGluR5a/b in cos cells (Tadokoro, et al. 1999).

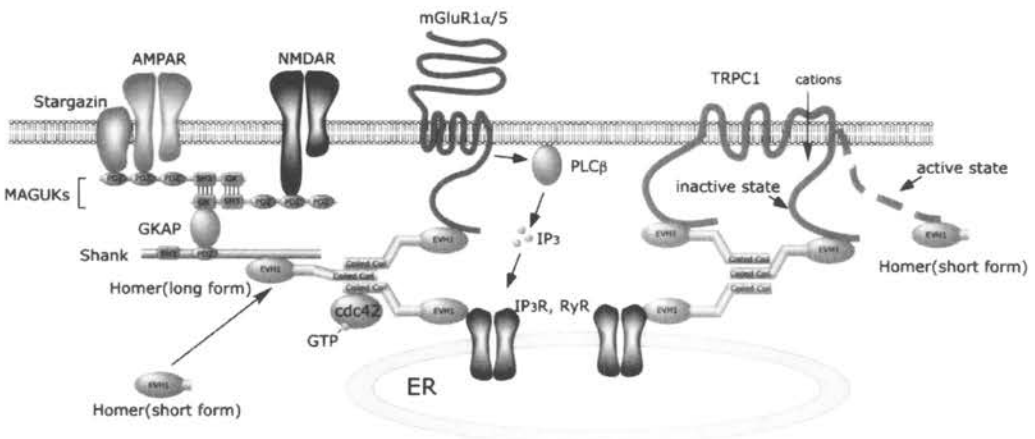


Figure 7. Schematic representation of the postsynaptic protein interactions that involve Homer proteins. The EVH1 domain of Homer proteins binds mGluR1a/5 (which in turn couple to phospholipase C; PLC), intracellular calcium channels (IP3R, RyRs), and PSD proteins (Shank) that interact with ionotropic glutamate receptors. *Adapted from Ehrengruber, et al 2004*

In cultured cerebellar granule cells, expressed mGluR5 is localized exclusively in the soma. Only upon co-expression of exogenous Homer 1b/c is the receptor targeted to dendrites, where it is present in clusters that co-localizalized with the

synaptic protein synaptophysin (Ango, et al 2000). Co-expression of Homer 1a non-specifically re-directs mGluR5 to both axons and dendrites. The authors concluded that long forms of Homer direct the targeting of Group I mGluRs to dendrites, the result of an "axon exclusion targeting signal".

A similar conclusion was drawn by Ciruela and colleagues working in cortical neurons. They found that only when mGluR1a is co-expressed with Homer 1c (a long form of Homer) does the receptor's expression extend into neurites, where it is synaptically localized with Homer (Ciruela, et al 2000). As Homer proteins are highly expressed in cerebellum (Homer 3) and cortex (Homer 1b/c; Homer 2a/b; Shiraishi, et al 2004) it is perplexing that receptor localization is dependent upon exogenous expression of Homer.

Homer also may play a role at the plasma membrane, where it is thought to be important in receptor clustering. While many studies examining a direct role for Homer in Group I mGluR clustering have been done in heterologous cells (Tu, et al 1999; Ciruela, et al 2000; Tadokoro, et al 1999), only within recent years has that finding been extended to neurons (Serge, et al 2002). When Homer 1b was exogenously expressed with mGluR5, both proteins were found to cluster in hippocampal neurons. The lack of mGluR5 clustering prior to exogenous Homer 1b expression suggested to the authors that endogenous Homer 1b was present at very low levels and could not induce mGluR5 clustering. This conclusion is at odds with other studies that have demonstrated that all (long) isoforms of Homer are robustly clustered at synapses in hippocampal cultures (Shiraishi, et al 2003;

Das and Banker, unpublished observations). Yet in keeping with the role of Homer and Group I mGluR clustering, Serge and colleagues also found that the lateral movements of exogneous mGluR5 (co-transfected with Homer 1b) were increased when the carboxyl domain of mGluR5 was truncated, compared to the co-expression of wild-type proteins. The C-terminus of mGluR5 contains the polyproline recognition motif that mediates its interaction with Homer. As a result, Homer 1b appears to play a role in anchoring the receptor to a particular subdomain in neurons and presumably helps contribute to its synaptic localization (Serge, et al 2002).

Thus, while many studies have established a role for Homer proteins in Group I mGluR trafficking, the conflicting results point to the complexity of this role in regulating receptor expression at the plasma membrane. These results also allude to the need to perform such trafficking assays in a system where both Group I mGluRs and Homer proteins are natively expressed. Such native systems would more likely utilize the same mechanisms for protein trafficking as would be found endogenously and thus would ensure more accurate, as well as consistent, results.

PDZ Domains

Named for the three proteins originally determined to possess these similar amino acid sequence motifs (**P**SD-95/**D**iscs-Large/**Z**ona-Occludens1), PDZ domains have been implicated in virtually every step of the protein trafficking

pathway. In some cases, the PDZ domain is integral to the expression of a protein on the cell surface, often helping to traffic it through the early secretory pathway (Fernandez-Larrea, 1999; Nakahama, et al 2002; Stricker and Huganir, 2004). In neurons, PDZ domain interactions are necessary for trafficking of several synaptic proteins to the cell surface (Standley, et al 2000; Sans, et al 2001). PDZ domains also play a part in polarized targeting within neuronal and epithelial cells (Rongo, et al 1998; Kaech, et al 1998; Xia, et al 2003) and even in localization to synaptic sites (Tejedor, et al 1997; Zito, et al 1997; Piccini and Malinow, 2002; Chang and Rongo, 2005). Protein localization can also depend on anchoring within the plasma membrane (Perego, et al 1999; Osten, et al 2000; Lazar, et al 2004). Another way of looking at this is that if a membrane protein is not stabilized at the cell surface, it can be endocytosed at an increased rate (Cao, et al 1999; Xiang, et al 2003). PDZ modules have been suggested to regulate both anchoring and endocytosis.

With this general background, I will next discuss two proteins that interact with mGluR1a via the receptor's PDZ binding motif: Tamalin and Shank.

Tamalin

Tamalin was first identified as an interacting partner for Group I and II mGluRs (Kitano, et al 2002). Tamalin is thought to function as a scaffolding molecule, and contains a number of motifs that mediate its ability to bind other proteins. These include a leucine zipper, a PDZ domain, a PDZ binding motif, and an SH3

binding site (Src holmology 3; PXXP) that is within an alanine/proline rich region. While mGluR1a was originally proposed to interact with tamalin via the receptor's PDZ binding motif, subsequent studies determined that this interaction also involves an upstream SH3-containing, proline-rich region (Hirose, et al 2004).

Since this original study, a number of other tamalin interacting partners have been identified. These include such scaffolding proteins as PSD-95 and Mint-2 – proteins with previously demonstrated roles in protein trafficking or anchoring (Kitano, et al 2003).

Tamalin may play a role in endocytosis by bringing the machinery necessary for endocytosis in proximity to mGluR1a. However, whether tamalin plays a definitive part in the trafficking of mGluR1a or other proteins is unclear. As there is some uncertaintly regarding this protein, I will try to describe what *is* known regarding Tamalin to better explain how it may putatively modulate mGluR1a trafficking.

After tamalin was cloned, it was found to be identical to GRASP, a scaffolding protein that binds GRP-1, a member of the cytohesin family of ARF-GEFs (guanine exchange factors for ADP ribosylation factors; Nevrivy, et al 2000). This protein was named "GRASP" for **GR**P-1 **as**sociated **p**rotein; herein I will refer to it as tamalin for the sake of clarity. Nevrivy and colleagues suggested that tamalin functioned as a scaffolding molecule due to the presence of a

number putative protein-protein interaction domains contained in this protein (Nevrivy, et al 2000; also Kitano, et al 2002).

The ARF-GEF family also includes Cytohesin-1, and ARNO (Cytoshesin-2), proteins that interact primarily with ARF6, a GTPase that has been found to function primarily between the endosome to plasma membrane (Donaldson and Jackson, 2000). The interaction with tamalin (or other such scaffolding proteins), may allow ARF-GEFs to interact with only those proteins in a particular subcellular domain. To that end, studies have demonstrated that the subcellular site of action for these GEFs is the plasma membrane where they have been implicated in ARF6 exchange.

In 2000, Mukherjee, et al implicated that ARF6 in β-arrestin-dependent receptor desensitization of the luteinizing hormone-choriogonadotropin receptor (LH-CGR), a GPCR. Activation of this receptor by its ligand (human choriogonadotropin, hCG) resulted in receptor downregulation independent of heterotrimeric G-proteins (Mukherjee, et al 2000). Since the study of LH/CGR by Mukherjee et al, a role for ARF6 (and its corresponding GEFs and GAPs) has been demonstrated in the desensitization of a number of GPCRs. These include the M2 muscarinic acetylcholine receptor, thyrotropin receptor, angiotensin type I receptor, vasopressin receptor, and β2-adrenergic receptor (Claing, et al 2001; Delaney, et al 2002; Houndolo, et al 2005; Lahuna, et al 2005). Therefore,

ARF6-dependent desensitization may prove to be a general mechanism utilized by GPCRs during receptor endocytosis.

As a result of the studies of cytohesins and ARF6 and their role in membrane trafficking, it is not surprising that Kitano and colleagues (2002) attempted to demonstrate a role for tamalin in Group I mGluR (mGluR5) trafficking. Endogeneous mGluR5 distribution was found confined to the soma in cultured neurons with little present in neurites. Expression of tamalin resulted in a redistribution of mGluR5 immunoreactivity throughout the processes. This suggested to the authors that tamalin is necessary for the trafficking of mGluR5 (and mGluR1a) into dendrites.

As a result of the findings that tamalin links mGluRs to ARF-GEFs of the cytohesin family, and since at least one family member has been shown to regulate GPCR endocytosis, it raises the possibility that mGluR1a endocytosis may be regulated in part due to its interaction with the scaffolding protein tamalin. Additionally, tamalin has the potential to regulate the postsynaptic localization of mGluR1a through its interactions with PSD95 and MINT2.

Shank

Shank is a synaptic scaffolding molecule that possesses multiple protein interaction domains including ankyrin repeats, an SH3 domain, a PDZ domain, several proline rich sequences and a self-association motif (Naisbitt, et al. 1999).

The PDZ domain of Shank binds the C-terminal PDZ interaction motif of the guanylate kinase-associated protein (GKAP), which in turn binds the Guanylate Kinase (GuK) domain of PSD95. One of the poly-proline sequences within Shank also interacts with Homer (Tu, et al., 1998, 1999). Another of Shank's proline motifs interacts with cortactin, a protein that crosslinks actin. Additionally, in heterologous cells, the PDZ domain of Shank was shown to bind directly to the C-terminal PDZ binding motif of mGluR5, an interaction which was then extended to mGluR1a (Tu, et al 1999). The importance of this interaction between metabotropic glutamate receptors and Shank has not been assessed in neurons. Thus, Shank could potentially link ionotropic and metabotropic glutamate receptors to the postsynaptic cytoskeleton.

Other Interacting Proteins

Recent studies have demonstrated that mGluR1a is capable of interacting with a number of other proteins including tubulin, $GABA_B$ receptors, adenonsine A1 receptors, muscarinic acetylcholine receptors (mAchRs), calcium channels, calcium sensing receptors (CaRs), Ephrin-B and protein 4.1G (Ciruela, et al 1999b; Tabata, et al 2004; Ciruela, et al 2001; Kitano, et al 2003; Calo, et al 2005; Lu, et al 2004). In most cases it is not known if metabotropic glutamate receptors and these proteins interact directly. Nor is there conclusive evidence that any of these proteins regulate mGluR1a trafficking.

Of possible relevance to my work, mGluR1a has been shown to heterodimerize with CaRs (Gama, et al 2001) in HEK-293 cells. However, the functional implications of this interaction have yet to be determined. While mGluR1a is a protein found in the somatodendritic domain (Martin, et al 1992; Baude, et al 1993), immunostaining shows that CaRs are localized to axon terminals (Ruat, et al 1995). Thus the likelihood or their interaction *in vivo* is still in question.

GPCR TRAFFICKING

A variety of G-protein coupled receptors are polarized when expressed in epithelial cells. These include the follicle stimulating, thyrotropin and luteinizing hormone receptors, thyrotropin releasing hormone, α_{2A}-adrenergic receptor, and M_3 muscarinic acetylcholine receptor (Keefer, et al 1994; Yeaman, et al 1996; Beau, et al 1998 and 2004; Saunders, et al 1998; Nadler, et al 2001; Chuang and Sung, 1998; Iverson, et al 2005). Some have also extended these studies into neurons as well (Wozniak and Limbird, 1998; Ghavami, et al 1999; Stowell and Craig, 1999; Jolimay, et al 2000; Francesconi and Duvoisin, 2002). Since the molecular determinants regulating the polarized distribution of these receptors have been identified in only a subset of cases (Beau, et al 1998 and 2004; Stowell and Craig, 1999; Francesconi and Duvoisin, 2002; Iverson, et al 2005), there are still many questions surrounding the mechanisms involved in the polarized targeting of GPCRs. Do all polarized GPCRs from the same family utilize similar targeting motifs? Are there sequence and/or structural similarities between these motifs? Continued investigations into this important family of receptors is needed before answers can be found.

The trafficking of metabotropic glutamate receptors has been examined in both polarized epithelial cells and neurons and below I will highlight those studies. Stowell and Craig (1999) proposed that mGluR7 has an axonal targeting signal, following chimeric studies with mGluR2 (which is dendritic in hippocampal

neurons). When the carboxyl tail of mGluR2 was exchanged for that of mGluR7 and the chimera expressed in hippocampal neurons, the mGluR2-tail7 receptor was expressed in both axons and dendrites. The problem with these results lies in their interpretation. A protein that is dendritic is expressed predominantly on the somatodendritic cell surface (Jareb and Banker, 1998). In the same manner, an axonal protein is present largely on the axonal cell surface (Sampo, et al 2003). When the distribution of either protein is disturbed as a result of mutation of targeting signals, mis-targeting usually results in an unpolarized distribution, that is the protein is expessed on *both* the dendritic and axonal cell surface. In the paper by Stowell and Craig, mGluR7 exhibited such a uniform expression pattern in hippocampal neurons. Thus it is a misnomer to consider that the receptor was specifically targeted to axons, as it was also present in dendrites. It is more likely that the mGluR2-tail7 construct was present in axons (and dendrites) due to the fact that its dendritic localization signal had been disrupted.

In MDCK cells mGluR7 is polarized to the basolateral domain (McCarthy, et al 2001). The carboxyl domain is important for surface expression on the basolateral surface but is not sufficient to target another unrelated protein (PLAP) to the basolateral domain. When expressed in neurons, both wild-type mGluR7 and the chimera PLAP-mGluR7tail were present along both axons and dendrites. The authors interpreted these results more accurately, indicating that the C-terminus of mGluR7 was inadequate to confer polarization to the axon.

mGluR1a targeting has also been compared to that of its shorter splice variant, mGluR1b, in chick retinal cells (Francesconi and Duvoisin, 2002). While mGluR1a was targeted to dendrites, the authors found mGluR1b only in axons. Their conclusion is that the RRKK motif in mGluR1b accounts for its axonal localization, one which is superceded by a more dominant dendritic targeting signal present in the long splice variant, mGluR1a (between amino acids 1011-1071). This result may pertain only to retinal cells, as mGluR1b has been shown to be present largely in dendrites in the rat hippocampus and cerebellum (Ferraguti, et al 1998; Mateos, et al 2000), a result I confirmed (data not shown).

Finally, mGluR1a was found to be differentially distributed at synapses in fusiform cells of the dorsal cochlear nucleus in basal, but not apical dendrites (Rubio and Wenthold, 1997). This distribution was specific for only a subset of glutamate receptor subunits (mGluR1a and GluR4), as all other subunits (including GluR2/3, and NR1) were present to the same degree in apical and basal dendrites. In a subsequent study, the authors also demonstrated an enrichment of mGluR1a and GluR4 in intracellular compartments (corresponding to the endoplasmic reticulum) in basal dendrites (Rubio and Wenthold, 1999). The authors suggest that the specific localization of some receptors points to a specialized mechanism that may preferentially synthesize, target and/or anchor various dendritic proteins from early in the biosynthetic pathway, until the receptor reaches the synapse.

In an effort to examine some of the mechanisms employed for the localization of dendritic proteins we chose to study the glutamate receptor mGluR1a in cultured hippocampal neurons. Chapter 2 of this thesis is a characterization of the pattern of expression exhibited by endogenous mGluR1a receptors in a subpopulation of hippocampal interneurons. Chapter 3 describes the role of protein interaction domains in regulating receptor polarity in dendrites and clustering at synapses. Chapter 4 summarizes the studies presented in this dissertation, critically evaluating the conclusions drawn, and touches upon future avenues of research.

CHAPTER 2

EXPRESSION OF mGluR1a IN CULTURED HIPPOCAMPAL NEURONS

INTRODUCTION

mGluR1a is a Group I metabotropic glutamate receptor that when triggered, results in the activation of phospholipase C and protein kinase C, as well as the release of Ca2+ from internal stores (Hermans and Challiss, 2001). Robust expression of mGluR1a has been found in the olfactory bulb, thalamus, hippocampus and cerebellum, where the receptor is present in the somatodendritic domain of cells (Martin, et al 1992; Baude, et al 1993; Shigemoto, et al 1997). At the synaptic level, mGluR1a is localized to the annulus surrounding postsynaptic densities (Baude, et al 1993; Lopez-Bendito, et al 2001 and 2002).

In situ hybridization and immunohistochemical analyses have both determined that while mGluR1a is not present in pyramidal neurons, it is expressed in a subpopulation of interneurons in the hippocampus (Martin, et al 1992; Baude, et al 1993; Shigemoto, et al 1997; Berthele, et al 1998; Ferraguti, et al 2004). Many of the mGluR1a-positive neurons also express somatostatin (Baude, et al 1993; Hampson, et al 1994).

The receptor is also expressed in several cell types that co-stain for other neuropeptides, in addition to cells that express somatostatin (Ferraguti, et al 2004). Many mGluR1a-positive/somatostatin-positive neurons are located in the stratum oriens and alveus while those neurons expressing other neuropeptides

are present in different layers within CA1 (Ferraguti, et al 2004). However, mGluR1a-positive/somatostatin-positive cells can also be found in layers CA3 and the dentate gyrus (Oliva, et al 2000).

In contrast to the expression pattern of mGluR1a, other Group I metabotropic glutamate receptors are more widely expressed in both principal and non-principal cells (Shigemoto, et al 1997; Ferraguti, et al 1998; Lopez-Bendito, et al 2002). The localization of all Group I mGluRs examined to date has found these receptors present in the somatodendritic domain of hippocampal neurons, consistent with a postsynaptic distribution (Baude, et al 1993; Lujan, et al 1996; Ferraguti, et al 1998; Lopez-Bendito, et al 2002). These findings illustrate that different neuronal subtypes may share common mechanisms in receptor localization.

Dissociated cell cultures from the hippocampus are a widely used system for studying neuronal development and synapse formation (Bartlett and Banker, 1984a and 1984b; Dotti, et al 1988; Craig, et al 1994 and 1996). The expression and distribution of ionotropic glutamate receptors in cultured hippocampal neurons have been extensively examined (Craig, et al 1993 and 1994), but the cells expressing mGluRs in these cultures have not been well characterized. A previous study determined that only a few neurons express mGluR1a in hippocampal cultures. In these cells, presumably interneurons, the receptor is restricted to the somatodendritic domain (Craig, et al 1993). It is still not known if

multiple mGluR1a-positive interneuron subtypes are present in hippocampal cultures, corresponding to those found *in situ* (Ferraguti, et al 2004). Nor is it known if the endogenously expressed receptor has a synaptic localization.

The goal of this study was to identify the neuron populations expressing mGluR1a in hippocampal cultures, to characterize their morphology, and to assess the subcellular localization of mGluR1a. In addition, we sought to examine whether mGluR1a exogenously expressed in hippocampal pyramidal neurons, exhibited characteristics similar to those of endogenous mGluR1a. We found that mGluR1a was endogenously expressed in two cell types with very different morphologies. One type expressed somatostatin, the other did not. In both cell types mGluR1a was restricted to the somatodendritic domain. Finally, live-imaging experiments demonstrated that when exogenously expressed in hippocampal cultures, GFP-mGluR1a was similarly restricted to dendrites where carriers could be found to traffic bi-directionally.

MATERIALS AND METHODS

Cell Culture and Transfection

Primary hippocampal neuronal cultures were prepared as described previously (Goslin, et al. 1998). Briefly, hippocampi were dissected from rats on embryonic day E18. These hippocampi were then dissociated and plated onto coverslips treated with poly-L-Lysine at a density of 50 cells/mm^2 and cultured over a monolayer of astrocytes. Cells were maintained in Neurobasal media supplemented with B27 and Glutamax. Immunostaining of cells for endogenous proteins was carried out in neurons that were between 21 and 24 days in culture. After 8-10 days in culture, neurons were transfected using Lipofectamine 2000 transfection reagent (Invitrogen).The distribution of expressed proteins was assessed using indirect immunofluorescence and live imaging, unless otherwise indicated.

Immunofluorescence

For most experiments cells were fixed with 4% Sucrose/4% Paraformaldehyde in 1x phosphate-buffered saline (PBS) for 20 minutes at room temperature. Coverslips were then permeabilized using 0.25% Triton X-100 reagent (Sigma) in 1x PBS for 5 minutes and washed three times in 1x PBS for 5 minutes per wash prior to being placed in blocking solution (250 microliters Gelatin [Sigma Cat#:G-7765] in 50 milliliters 1x PBS) for 30 minutes. This solution was used to dilute all antibodies, as well as to wash cells after antibody treatment. Neurons were

stained for the presence of endogenous mGluR1a using a polyclonal antibody directed against a portion of the receptor's intracellular carboxyl domain (Chemicon) at a dilution of 1:150. In order to assess the cell surface population of mGluR1a, an antibody directed against the receptor's extracellular amino-terminus (kindly provided by Dr. R. Shigemoto) was used at a dilution of 1:50. Neurons were co-stained for either somatostatin (Biomeda, V1169) or MAP2 (Sigma clone HM-2) using monoclonal antibodies directed against the respective protein.

Following primary antibody application, neurons were incubated with biotinylated donkey anti-rabbit secondary antibody (West Grove, PA) for 1 hour followed by three washes in blocking medium for 5 minutes per wash. In cases where neurons were co-stained, an Alexa 633 (Eugene, Oregon) conjugated goat anti-mouse antibody was applied for 1 hour followed by washing as described above. A Cy3 conjugated to streptavidin (Vector Laboratories, Burlingame, CA) was then placed on cells at a dilution of 1:10,000 for 15-30 minutes, followed by three washes in blocking medium for 5 minutes each. Coverslips were rinsed in dH_20 prior to being mounted on slides using ProLong Gold Anti-Fade reagent (Molecular Probes).

DNA Constructs

The pJPA expression vector was used for all constructs in this study (J.Adelman, OHSU). Wild-type mGluR1a was obtained from G. Westbrook (Vollum Institute,

OHSU). A sequence encoding GFP (cyan fluorescent protein) was inserted into the wild-type cDNA shortly following the predicted signal sequence (Masu, et al 1991) at the Eco47III site. The cDNA construct was checked by restriction enzyme analysis and sequencing.

Microscopy

A wide-field Leica microscope (DMIRBE) equipped with a 16x and 63x objective (Numerical Aperture = 0.50 and 1.32, respectively) was used to acquire all images. Images were acquired using a Roper Scientific (formerly Princeton Instruments, Inc.) Micromax 5MHz-1300Y camera equipped with a Sony Chip ICX061 (1300 x 1030 Interline CCD camera) controlled by MetaMorph image acquisition and analysis software (Universal Imaging Company, Downingtown, PA). Live imaging was performed using a Yokogawa CSU-10 Nipkow spinning disk confocal head mounted on a Nikon TE-2000 microscope. For illumination, the 488 or 514 nm laser lines were selected from a Coherent Laser INNOVA 70C krypton-argon laser using the NEOS AOTF. Images were acquired on a Hamamatsu Orca ER camera controlled by QED software (Roper Scientific). Temperature was maintained at 37 degrees during image acquisition. To quantify transport events, the kymograph drop-in function of the METAMORPH IMAGING SOFTWARE (Universal Imaging, Downingtown, PA) was utilized. Briefly, lines were drawn along the axis of individual neurites, and the kymograph function was used to find the brightest pixel along a 10-pixel line perpendicular to the axis of the neurite. These values were then plotted for each frame, with time on the *x* axis

and position along the neurite on the *y* axis. Thus, moving vesicles appeared as diagonal lines whose slopes were a measure of rate and direction of transport (with positive slope corresponding to anterograde transport).

RESULTS

mGluR1a was expressed in two distinct cell types in hippocampal neuronal cultures

A previous brief report from this lab found that mGluR1a was expressed in a small percentage (less than 1%) of hippocampal neurons (Craig, et al 1993), as has been described *in situ* previously (Baude, et al 1993). While the latter study suggested that mGluR1a-positive neurons exclusively co-localized with somatostatin, recent findings indicate that mGluR1a may be expressed in a number of different interneuron cell types, not all of which co-localize with somatostatin (Ferraguti, et al 2004).

To identify mGluR1a-expressing cells in culture, we immunostained dissociated hippocampal neurons and evaluated both the neuronal morphology and expression patterns of mGluR1a-positive neurons. We used low-density hippocampal cultures, which have been used before in order to identify and classify GABAergic interneurons based on dendritic morphology (Benson, et al 1994).

Two predominant types of cells were immunopositive for mGluR1a; representative examples of each are iillustrated in Figure 1. Type I mGluR1a neurons had very long dendrites which emanated from either side of the soma (up to 180 degrees away from one another) and meandered around the cell's

soma and nearby cells. There were approximately 2-4 dendrites, on average, per cell and branching often occurred very distally in the primary dendrites – sometimes 100-200 μm away from the point at which the primary dendrite emanated from the soma (Figure 1a-c). Additionally, Type 1 cells expressed mGluR1a at high levels.

In Type II mGluR1a neurons, the dendrites were slightly shorter and there were more primary dendrites per cell --- 3-6 on average. Dendrites were oriented radially from the soma and branching of primary dendrites tended to occur between 50-100 μm from the soma, much closer than in Type I cells (Figure 1b, d).

The subcellular localization of mGluR1a in dendrites was also different in the two cell types. In Type I cells, mGluR1a was distributed in the soma and throughout all the cells dendrites in a uniform, slightly granular pattern (Figure 2a, b). However, in Type II cells, staining was concentrated in distinct oblong patches all along dendrites (Figure 2c, d). The somatic staining of Type II cells also appeared to be (slightly) greater than Type I cells (Figure 1b, d).

<u>mGluR1a-positive patches in Type II cells were largely present on the cell surface</u>

The patches of mGluR1 staining in Type II cells could be on the cell surface, possibly at synapses, or could represent an intracellular pool of receptor. In

order to preferentially stain the cell surface population of mGluR1a receptors, we utilized an antibody directed against the extracellular amino-terminus (kindly provided by Dr. R. Shigemoto) and stained cultures following fixation but without permeabilization. While fixation often results in slight permeabilization of the plasma membrane, nevertheless, this technique should bias staining towards receptors present on the cell surface.

Using the N-terminal antibody, we obtained results similar to those with the C-terminal antibody, under conditions that detect both intracellular and cell surface receptor (Figure 1). In particular, Type II neurons showed distinct accumulation of mGluR1a in patches along dendrites (Figure 3a,b). Although staining of Type II neurons was more robust with the N-terminal antibody than with the antibody against the C-terminus, Type I cells appeared dimmer following staining with the N-terminal antibody. These observations raise the possibility that the proportion of intracellular mGluR1a is greater in Type I cells than Type II cells.

Type II mGluR1a neurons express somatostatin

Past studies have identified distinct populations of mGluR1a expressing interneurons, based on their expression of different peptides (including somatostatin, parvalbumin and vasoactive intestinal peptide; Ferraguti, et al 2004). We next asked whether one or both of the mGluR1a cell types identified in culture co-stained for somatostatin (using the antibody against the C-terminal domain).

Our results indicated that Type I mGluR1a-expressing neurons were immunonegative for somatostatin (Figure 4a-c). In Figure 4a-c, the mGluR1a expressing neuron (arrow) did not express somatostatin, although a nearby cell (arrowhead) that did not express mGluR1a robustly expressed the neuropeptide (Figure 4b). We found only one instance of a Type I cell that co-expressed somatostatin (data not shown). In contrast, Type II mGluR1a cells were somatostatin-positive (Figure 4d-f). This was true for all Type II cells examined. These results demonstrate that mGluR1a expressing neurons, in addition to having varied morphologies and receptor expression patterns, also express different neuropeptides making these neurons readily identifiable.

mGluR1a expression is restricted to the cell body and dendrites

To determine whether mGluR1a was polarized to the dendrites, hippocampal neurons were fixed, permeabilized and then double-stained with antibodies directed against the C-terminus of mGluR1a and MAP2, a microtubule associated protein whose staining is restricted to the soma and dendrites of neurons. In both Type I and Type II mGluR1a positive cells, mGluR1a staining was present only in neurites that were also immunopositive for MAP2 (Figure 5b-c;e-f). Many of these dendrites had no fine axonal processes crossing over them (see phase images, Figure 5a, c), further indicating that the mGluR staining must be associated with dendrites. In higher magnification images, the base of the

axon could often be identified based on the decrease in MAP2 staining. mGluR1a staining also ceased at the base of the axon. (see arrow Figure 5g-j).

Exogenously expressed mGluR1a trafficks bidirectionally within dendrites

The results presented so far indicate that endogenous mGluR1a is expressed within interneurons only in the somatodendritic domain. Previous studies have demonstrated that other members of the Group I mGluR family are expressed within pyramidal neurons, where they are also present in the somatodendritic domain (Shigemoto, et al 1997; Ferraguti, et al 1998; Lopez-Bendito, et al 2002). This suggests that both principal and non-principal cells in the hippocampus are capable of selectively localizing mGluRs to the appropriate domain.

In order to determine if exogenously expressed mGluR1a is capable of being appropriately targeted to the somatodendritic domain in hippocampal pyramidal neurons, we transfected hippocampal neurons with GFP-mGluR1a so that we could visualize carrier dynamics within living neurons. Figure 6a illustrates a pyramidal neuron (identified based on morphology) whose dendrites were imaged to generate a movie, thus identifying moving carriers. An axon could not be identified based on GFP fluorescence indicating that even when exogenously expressed mGluR1a is present only in dendrites (see also Chapter 3).

To quantify the transport of carriers, kymographs were made from the movies and vesicle position along the dendrite (y-axis) was tracked as a function of time

(x-axis). The resulting kymograph (Figure 6b and c) was used to determine the velocity of the moving carriers. Diagonal lines with a positive slope indicate carriers moving away from the soma. Those with a negative slope show movement toward the soma. Both anterograde and retrograde carriers were found to have a velocity of 0.35, slightly slower than transport observed for other carriers undergoing microtubule based transport (Burack, et al 2000; Silverman, et al 2001).

DISCUSSION

In this study, we characterized the population of mGluR1a-positive interneurons in low density hippocampal cultures. Our results indicate that there are two types of mGluR1a-positive cells that are distinguishable based on dendritic morphology and receptor expression pattern. Type I cells possess long dendrites which branch distally and express mGluR1a in a more uniform manner along dendrites. Type II cells have somewhat shorter denrites that branch closer to the soma and express this receptor in distinct patches throughout dendrites. All mGluR1a-positive cells were found to express the receptor only in the somatodendritic domain. Only Type II cells co-expressed the neuropeptide somatostatin.

In vivo, mGluR1a-positive cells from the stratum oriens and alveus stain very brightly for the glutamate receptor, compared to other areas in CA1 and virtually all of these neurons also express somatostatin (Ferraguti, et al 2004). However, in hippocampal cultures, the most brightly stained mGluR1a-positive neurons, were found to be devoid of somatostatin. Hippocampal cultures consist of neurons from all regions of the hippocampus, including CA1, CA3 and the developing dentate gyrus. Since only the CA1 was characterized in the study by Ferraguti and colleagues(2004), it may be that the interneurons in other parts of the hippocampus that express mGluR1a (Baude, et al, 1993; Shigemoto, et al 1997; Lopez-Bendito, et al 2002; Oliva, et al 2000) account for one or both of the

subtypes of neurons we have described in our study. Dissociated cultures do not allow us to determine the original location from which the neurons are derived.

A transgenic line of mice that expresses GFP within inhibitory neurons (under control of the murine GAD1 promoter) was found to also co-express both somatostatin and mGluR1a in the hippocampus (Oliva, et al 2000). It would be useful to use such cells in dissociated culture as they would be readily identifiable, even without immunostaining. Although only a small percentage of somatostatin-positive cells from the stratum oriens and alveus in CA1 were also found to express GFP, numerous GFP-expressing cells in other regions in the hipocampus could be found. Thus these mice might prove a useful means to study both the morphological and underlying molecular differences between mGluR1-positive neurons that express somatostatin and those that do not.

Our live imaging experiments support the notion that dendritic proteins are selectively targeted to the somatodendritic domain and do not traffic within axons. A previous study from this lab demonstrated that transferrin receptor, a single-pass dendritic protein, was selectively transported into dendrites (Burack, et al 2000). The results from the current study extend those findings to include the multi-pass receptor mGluR1a, which was also found to traffic exclusively with dendrites. Although the velocity of mGluR1a carriers was low, this may be due to the fact that these neurons were imaged at steady-state (24 hours after transfection) when the labeled population would include both Golgi-derived and

endocytic carriers. Golgi-derived carriers often move at a greater velocity than endosomes (G. Glover, unpublished observations; Prekeris, et al 1999). Regardless, all carriers, whether Golgi-derived or endosomal, were restricted to the somatodendritic domain.

Our results support findings *in situ* that have found that only a small fraction of cells express mGluR1a, indicating that the culture system is an accurate model of some hippocampal neuronal subtypes (Baude, et al 1993; Craig, et al 1993). One exception is pyramidal cells, which are not easily distinguishable in cultures and do not possess a similar morphology as in vivo. In contrast, these results (and former studies) have found that GABAergic populations of neurons are more easily distinguishable from one another (Benson, et al 1994). In the case of Type I and Type II mGluR1a-positive interneurons, their dendritic morphologies were very different and recognizable in our cultures.

These cultures provide a useful means by which one could examine a number of features regarding both mGluR1a-positive cells, in addition to other neuronal characteristics. Future experiments should allow us to determine if patches in Type II cells are synaptic based on co-localization with pre- and postsynaptic markers. Furthermore, one could utilize these cultures to examine a number of cell types which are known to have different molecular identities.

Figure 1. In hippocampal neurons, endogenous mGluR1a is expressed in cells with two distinct morphologies. (a,b) When hippocampal neurons were stained for endogenous mGluR1a using an antibody directed against the carboxyl domain of mGluR1a, two different cell types were found, each with a different pattern of expression for the receptor. Phase (a) and fluorescence (b) images illustrate two such cells located next to one another. On the left, a cell with long dendrites that meander around nearby cells expresses mGluR1a in a more uniform pattern throughout its processes. Additionally, the dendrites do not branch extensively from the primary dendrite close to the soma (Type I). On the right, a cell with shorter dendrites expresses mGluR1a in distinct "patches" along dendrites, although at this magnification, the patches are not readily apparent (Type II). Nearby neurons that do not express mGluR1a serve as a measure of antibody background. Background staining is present in cell bodies but not dendrites. **(c,d)** Two additional examples of each cell type are presented. **(c)** A Type I cell with meandering dendrites. **(d)** A second example of a Type II mGluR1a expressing neuron. At this higher magnification, the patchy nature of the mGluR1 staining is more apparent. These cells were fixed and stained on day 21. Arrows indicate dendrites from Type I cells, and arrowheads point to Type II dendrites. Scale bars: (a, b): 15 μm; (c, d): 15 μm

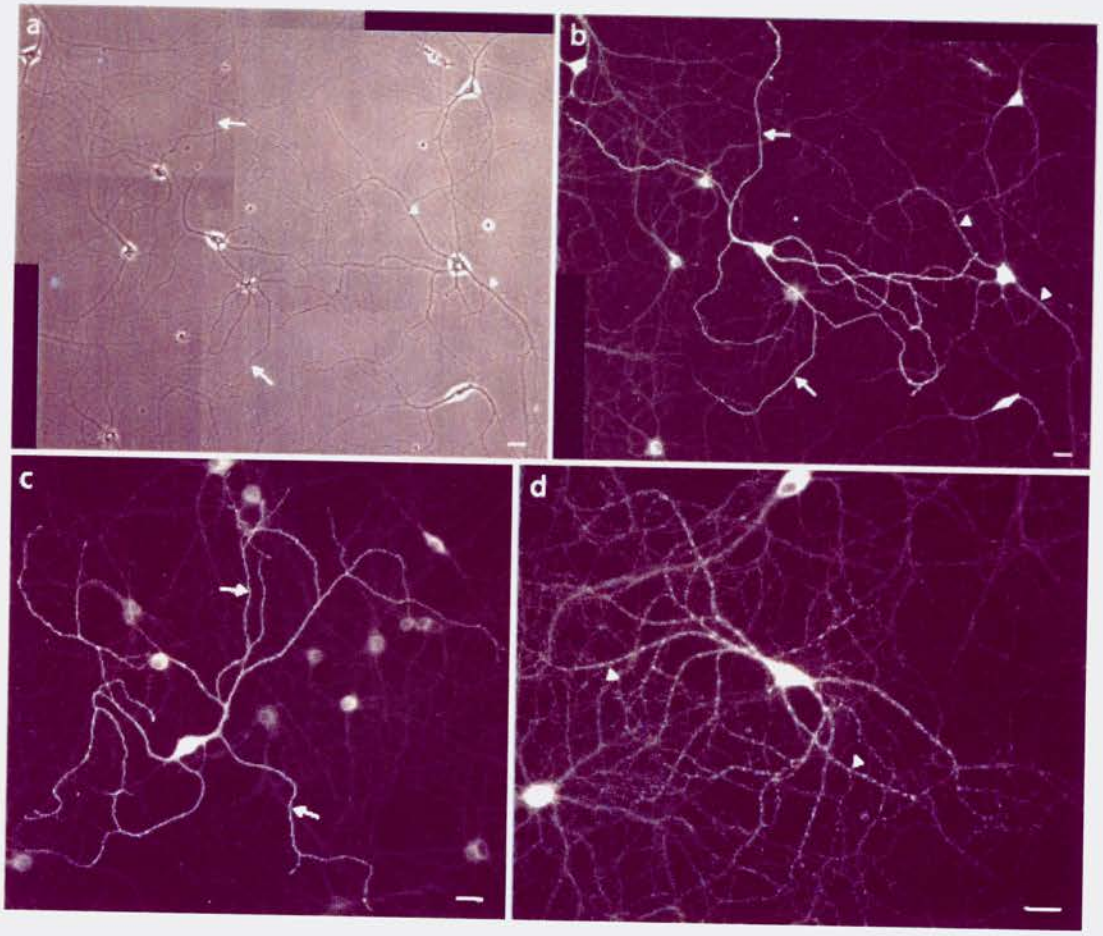

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Figure 2. High magnification images of mGluR1a staining in dendrites of Type I and Type II cells. **(a, b)** Dendritic segments from a Type I cell demonstrate a predominantly uniform expression pattern. **(c, d)** Dendrites from a Type II cell show mGluR1a localized in "patches". There is little staining for mGluR1a between patches. These cells were fixed and stained on day 21. Scale bar: 5 μm

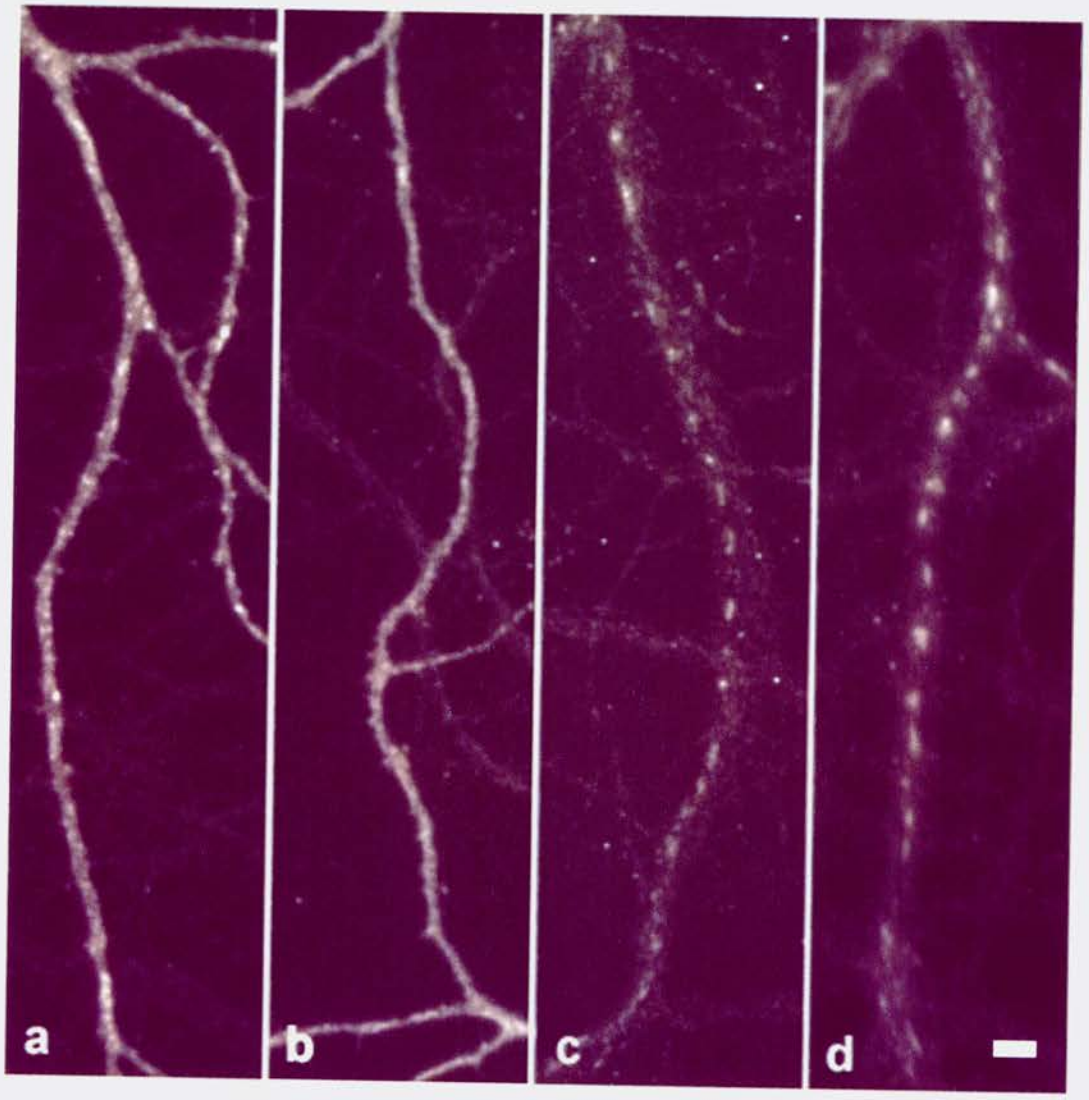
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Figure 3. The punctate distribution of mGluR1a on Type II neurons is more prominent under conditions that predominantly stain cell surface receptor. **(a)** The N-terminal antibody stains an mGluR1a expressing cells in a similar manner to those illustrated in Figures 1-2. Cell surface mGluR1a appears to be robustly expressed both in patches and on the soma. Nearby neurons not expressing the receptor have some fluorescence in cell bodies, but not to the same degree as the mGluR1a-expressing cell. **(b)** Higher magnification views show patches more clearly. Cells were fixed and incubated with an antibody directed against the N-terminus of the receptor, located extracellularly. This was done *without* any permeabilization to cells. Scale bars: (a): 15 μm; (b): 5 μm

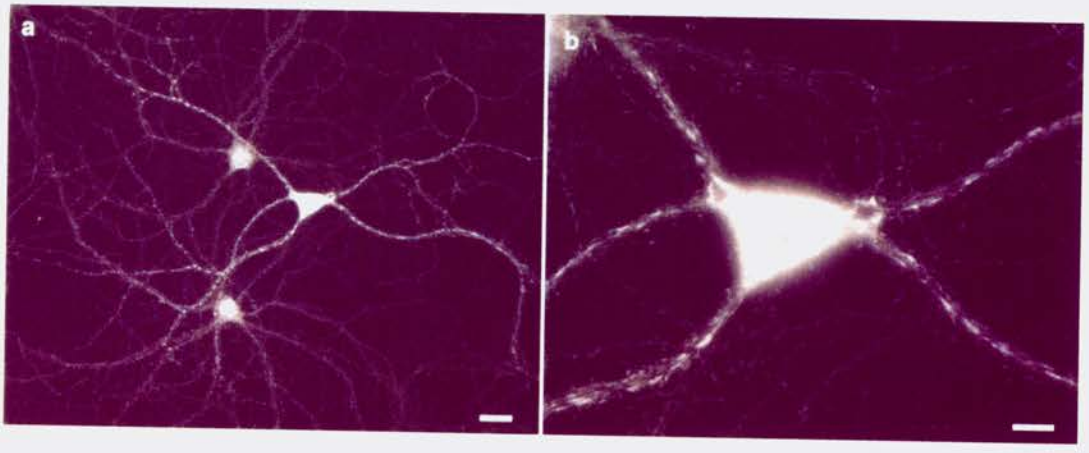
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Figure 4. Type II mGluR1a neurons express somatostatin. **(a)** A typical Type I cell (arrow), with a uniform mGluR1a expression pattern of mGluR1a in the soma and dendrites. The somata of mGluR1a-negative cells are also visible due to background staining. **(b)** A higher magnification view of the mGluR1a expressing cell and a second cell (arrowhead) that does not express the receptor. **(c)** Somatostatin staining reveals expression in the lower cell that does not express mGluR1a, but not in the Type I neuron. **(d-f)** A Type II mGluR1a expressing neuron co-expresses somatostatin. **(d)** A representative Type II mGluR1a expressing cell, showing patchy expression pattern in dendrites. **(e)** A higher magnification view of the mGluR1a staining that also shows abundant somatostin expression in **(f).** Cells were fixed and permeabilized prior to staining with an antibody directed against the carboxyl domain of mGluR1a and somatostain. Arrows (a-c) point to mGluR1a-positive neurons, arrowheads (a-c) to mGluR1a-negative neurons. Scale bars: (a):20 μm; (b, c):10 μm; (d):20 μm; (e, f):10 μm;

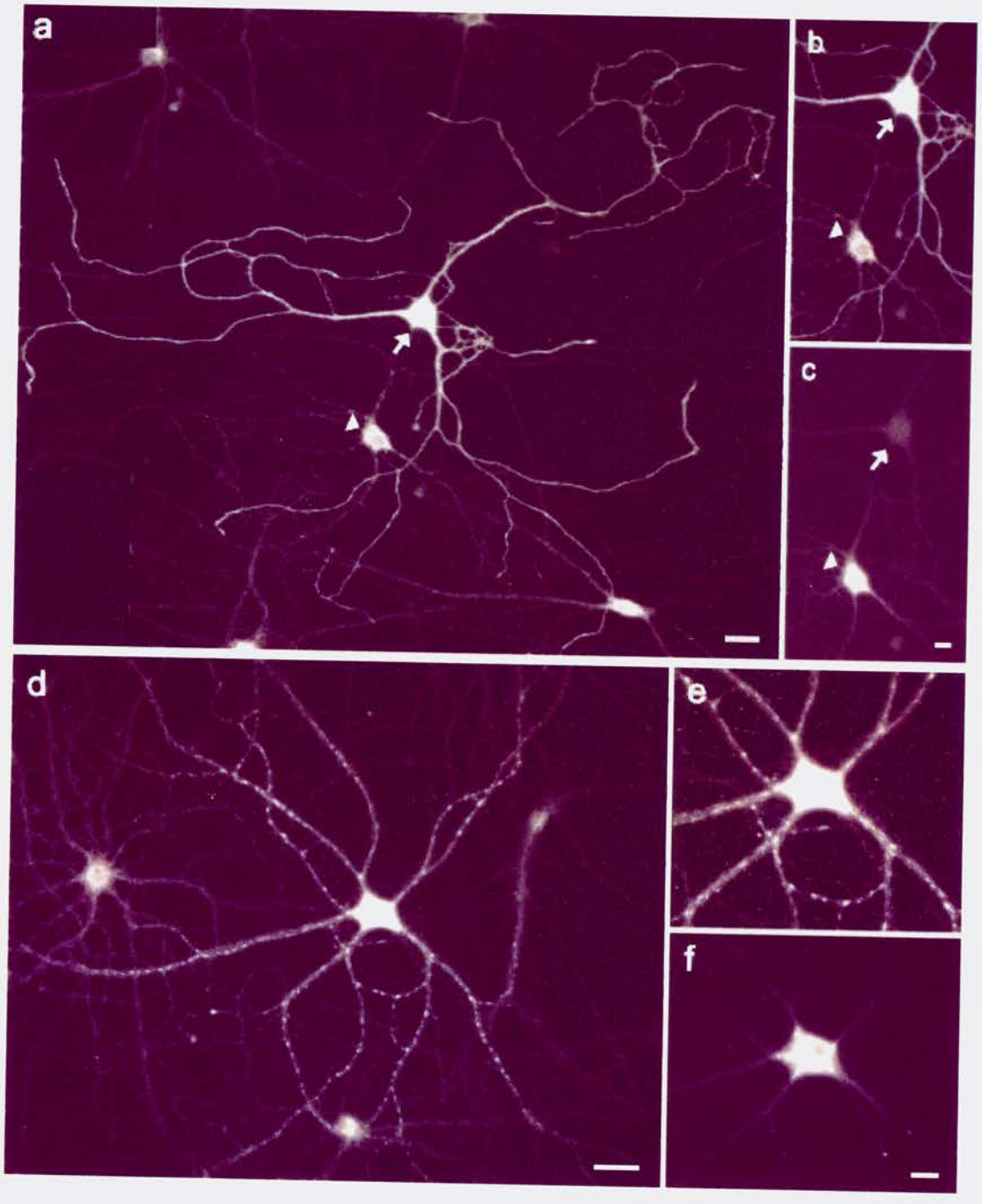
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Figure 5. mGluR1a co-localizes with the dendritic marker MAP2 in both Type I and Type II cells. (a-c) Indicated is an example of a Type I mGluR1a expressing neuron. **(a)** Phase image indicates processes from all cells surrounding the mGluR1a stained cell. **(b)** In this Type I cell a uniform mGluR1a expression pattern is apparent in the soma and dendrites. **(c)** MAP2 co-staining illustrates dendrites from this neuron. **(d-f)** An example of a Type II mGluR1a expressing neuron is presented in the following figures. **(d)** Phase image indicates processes from all cells surrounding the mGluR1a stained cell. **(e)** In this Type II cell mGluR1a immunostaining is apparent in the soma with a patchy expression pattern in dendrites. **(f)** MAP2 co-staining illustrates dendrites from this neuron. mGluR1a-positive neurites also stain for MAP2. Open arrowheads indicate dendritic processes (for both Type I and Type II mGluR1a expressing neurons). **(g-j)** At higher magnification the origin of the axon can be identified by the loss of MAP2 staining. mGluR staining stops at about the same location. Cells were fixed and permeabilized prior to staining with an antibody directed against the carboxyl domain of mGluR1a and MAP2. Arrows point to axons emanating from mGluR1a expressing cells, arrowheads to dendrites. Scale bars: (a-f): 15 µm; (g-j): 5 µm;

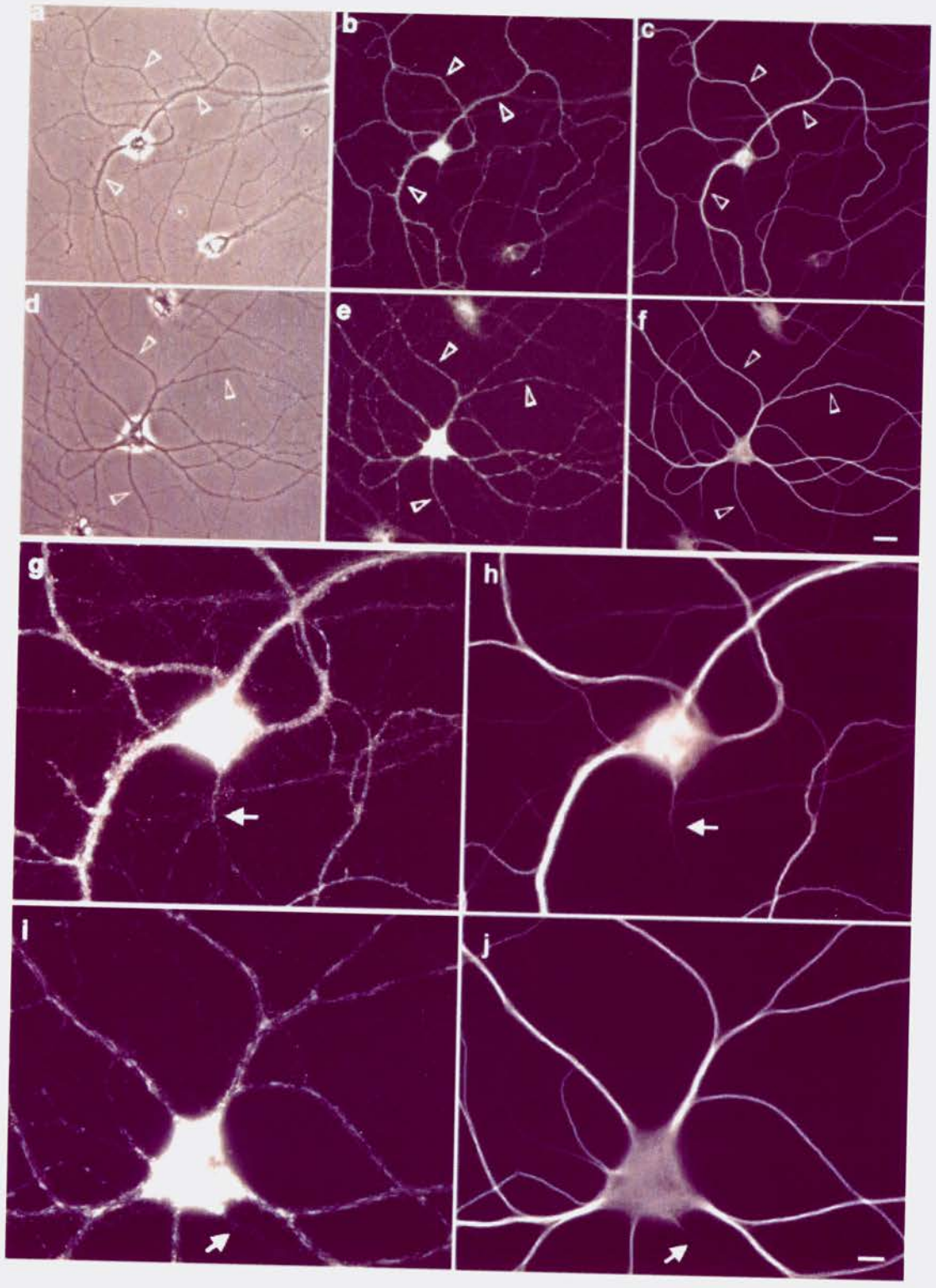
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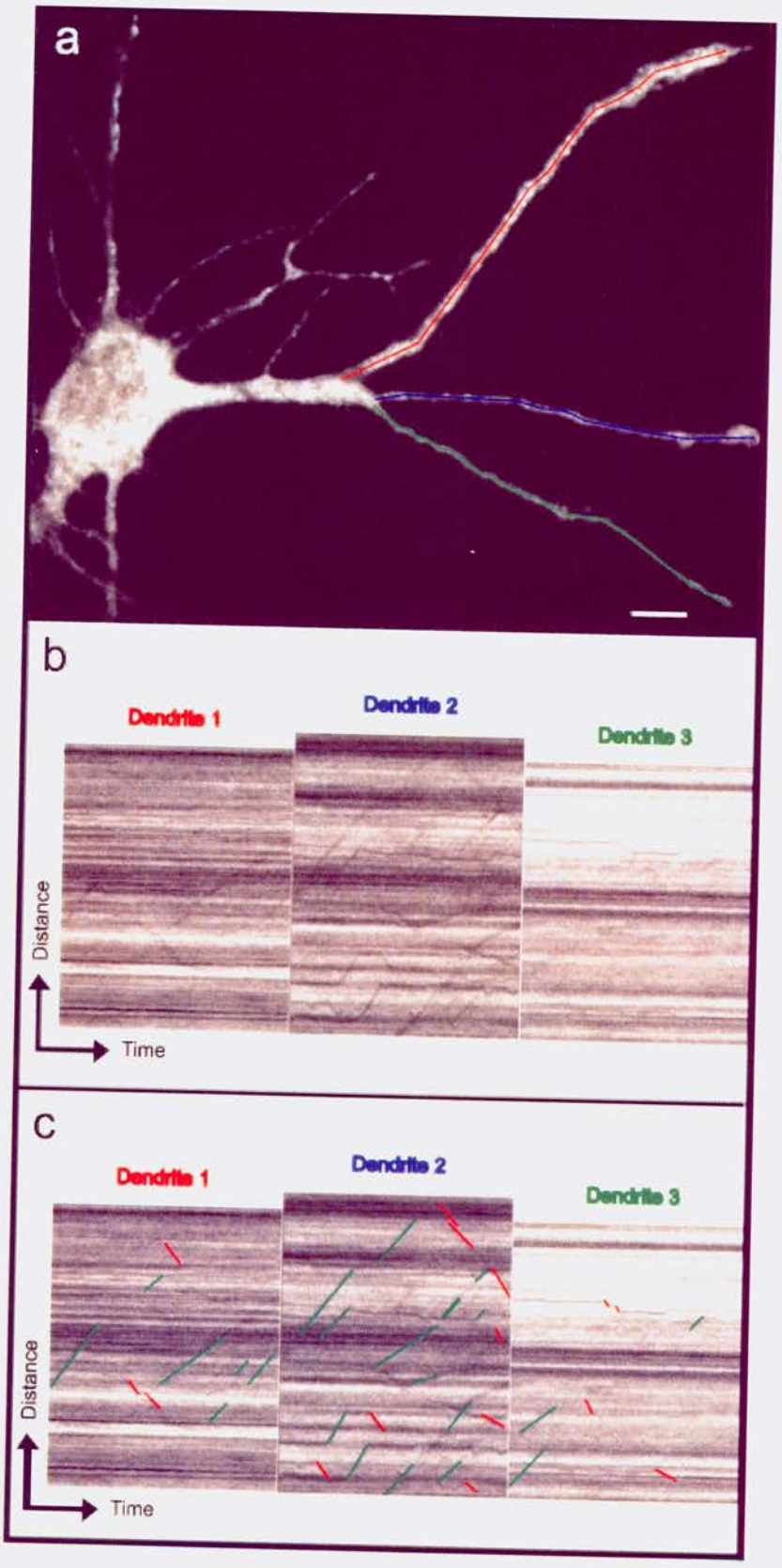
Figure 6. Exogenously expressed GFP-mGluR1a trafficks only within dendrites of pyramidal neurons (a) A pyramidal cell expressing mGluR1a was imaged and exhibited fluorescence within dendrites. Lines along dendrites correspond to the kymographs below. (b) Carrier movement was analyzed using kymographs to determine the distance traveled (y-axis) versus time (x-axis). Lines with a positive or negative slope indicate moving carriers traveling anterogradely or retrogradely, respectively. (c) Lines were drawn along those lines with a slope to calculate the velocity of carriers. Green lines correspond to anterogradely moving carriers; red lines indicate retrogradely moving carriers. Hippocampal neurons were transfected at 9 days in culture with GFP-mGluR1a and imaged using a spinning disk confocal after 24 hours. A 53 second movie was acquired that encompassed the dendrites indicated were then made so that carrier velocity could be determined using kymograph analysis (see Methods). Scale bar in (a): 5 μm.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Dendrite 3
Distance
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CHAPTER 3

THE ROLE OF PROTEIN INTERACTION MOTIFS IN REGULATING THE POLARITY AND CLUSTERING OF mGluR1a

INTRODUCTION

Protein trafficking and protein localization are key factors in nearly every aspect of neuronal function. For example, many channels and receptors are polarized to either dendrites or axons and further restricted to specific subdomains within the plasma membrane, such as postsynaptic sites or presynaptic terminals. While the polarization of such proteins is integral for neuronal communication, the specific localization of membrane proteins to either the axonal or dendritic domains facilitates a number of other important cellular functions including cell survival, the establishment of connections between neurons, cell patterning during cortical development, axo-dendritic outgrowth, and synapse assembly (Rakic and Caviness, 1995; Curran and D'Arcangelo, 1998; Skutella and Nitsch, 2001; Davies, et al 2003; Kim and Chiba, 2004; Cline, 2005).

Trafficking of polarized proteins to the nerve cell surface is thought to involve sorting of proteins into specific carriers along the biosynthetic and endocytic recycling pathways as well as selective microtubule-based transport to ensure carriers reach their correct destination (Craig and Banker, 1994; Horton and Ehlers, 2004). Following insertion into the plasma membrane, interaction with scaffolding proteins may refine protein localization to specific subdomains and regulate endocytosis (Sheng and Sala, 2001; McGee and Bredt, 2003).

By expressing mutant proteins in cultured nerve cells or transgenic animals, sequences that govern localization to dendrites have been identified in the cytosolic domains of a number of proteins, including ion channels and cell adhesion molecules (West, et al 1997; Jareb and Banker, 1998; Silverman, et al 2005; Rivera et al 2003; Lim, et al 2000; add Mitsui, et al 2005). Based on parallels with protein sorting in epithelial cells (Folsch, et al 1999), it is thought that these signals interact with protein adaptors to govern packaging into dendritic transport carriers. As no one has yet identified the adaptors that mediate dendritic sorting, by comparison, little is known about the mechanisms that underlie the localization of neurotransmitter receptors to dendrites. One difficulty is that many receptors, including most ionotropic receptors, are multi-subunit proteins. When mutant subunits are expressed in neurons, they form multimers with endogenous subunits that may contain sufficient information to correctly target the resulting complex (Ruberti and Dotti, 2000; Chang and Rongo, 2005).

This study concerns the localization of mGluR1a, a member of the Group I metabotropic glutamate receptor family that mobilizes intracellular Ca^{2+} stores and activates downstream effectors via PLC and PKC, in response to glutamate binding (Hermans and Challiss, 2001). There are several reasons why we chose to focus on mGluR1a. First, its distribution in neurons has been well characterized. mGluR1a is localized in dendrites to the annulus surrounding the postsynaptic density. Additionally, in the hippocampus, mGluR1a is only

expressed within a subpopulation of interneurons (Baude, et al. 1993; Ferraguti, et al 2004). Moreover, it is an easier receptor to study using an expression system because it exists as a homodimer, and cannot form heterodimers with any other mGluRs that are expressed in pyramidal cells (Romano, et al 1996; Robbins, et al 1999). Therefore, expressed mGluR1a does not need to contend with endogenous protein, which could potentially interact with expressed receptor, thereby affecting its cellular localization.

Finally, several interacting proteins have been identified that have been hypothesized to regulate the trafficking and localization of mGluR1a. The first of these proteins to be identified, Homer, is a scaffolding protein that links membrane receptors and intracellular signaling complexes (Brakeman, et al 1997; Tu, et al. 1998 and 1999; Xiao, et al. 1998; Yuan, et al 2003). mGluR1a interacts with members of the Homer family of proteins via a polyproline motif in it's carboxy terminus. In heterologous systems, co-expression of Homer with mGluR1a leads to receptor clustering (Tadokoro, et al. 2000; Ciruela, et al 2000). In cultured cortical and cerebellar neurons, Homer expression has been implicated in regulating the trafficking of receptors into neurites (Ango, et al. 2002; Ciruela, et al. 2000). Two other proteins, Tamalin and Shank, interact with mGluR1a via its PDZ binding domain (Tu, et al 1999; Kitano, et al. 2002). Tamalin binds to a number of other proteins known to play a role in trafficking to and from the plasma membrane including the scaffolding proteins PSD-95 and MINT2 (Kitano, et al. 2003). There is also evidence in cortical neurons that

Tamalin regulates Group I mGluR trafficking to dendrites (Kitano, et al 2002). Shank, which also interacts directly with Homer and cortactin, is thought to be involved in tethering receptors and other components of the postsynaptic scaffolding complex to the underlying cytoskeleton. All three of these mGluR1a binding proteins -- Homer, Tamalin, and Shank – are expressed in hippocampal pyramidal neurons (Tu, et al 1998; Naisbitt, et al 1999; Kitano, et al 2002).

In the current study, we first show that when expressed in cultured hippocampal neurons, mGluR1a is polarized to dendrites and clustered synaptically. We then sought to understand how protein interactions mediated by the Homer-binding and the PDZ-binding domains contribute to the localization of mGluR1a. We found that mutating the polyproline sequence that mediates an interaction with the Homer family of proteins eliminated receptor clustering at synaptic sites, but did not affect its dendritic localization. Deleting the PDZ binding domain did not affect either dendritic localization or receptor clustering. Deleting the entire cytoplasmic tail of mGluR1a only modestly reduced its dendritic polarity, but appending the cytoplasmic tail was sufficient to target an otherwise unpolarized protein to the dendrites.

MATERIALS AND METHODS

Cell Culture and Transfection

Primary hippocampal neuronal cultures were prepared as described previously (Goslin, et al. 1998). Briefly, hippocampi were dissected from rats on embryonic day E18. These hippocampi were then dissociated and plated onto coverslips treated with poly-L-Lysine at a density of 50-100 cells/mm^2 and cultured over a monolayer of astrocytes. Cells were maintained in Neurobasal media supplemented with B27 and Glutamax. After 8-10 days in culture, neurons were transfected using Lipofectamine 2000 transfection reagent (Invitrogen). The distribution of expressed proteins was assessed using indirect immunofluorescence, unless otherwise indicated.

DNA Constructs

The pJPA expression vector was used for all constructs in this study (J.Adelman, OHSU). Wild-type mGluR1a was obtained from G. Westbrook (Vollum Institute, OHSU). A sequence encoding CFP (cyan fluorescent protein) was inserted into the wild-type cDNA shortly following the predicted signal sequence (Masu, et al 1991) at the Eco47III site. mGluR1a(ΔCT) was made by truncating the wild-type mGluR1a 8 amino acids following the predicted 7th transmembrane domain. mGluR1a(ΔPDZ) was truncated at amino acid 1196, which removed the type I PDZ binding motif (AA1197-AA1199) which is the predicted Tamalin interaction domain (Kitano, et al 2002). The mGluR1a mutant that was unable to interact

with endogenous Homer -- mGluR1a(F1156R) was made by mutating amino acid 1156 from phenylalanine to arginine (Tu, et al 1998). pPJPA5-CD8α wild-type cDNA was also manipulated to make a truncated CD8α that ended 6 amino acids after the transmembrane domain (amino acids 1-216). The wild-type mGluR1a carboxy terminus was PCR'd from the original mutant constructs (see above) and inserted in frame with pJPA5 CD8α. PCR products consisting of the various mGluR1a carboxy termini were digested with AgeI and XbaI and subcloned into the pJPA5 CD8α vector to generate the following chimeric construct: pJPA5 CD8α-mGluR1a(wild-type). The entire segment generated by PCR of all cDNA constructs were checked by restriction enzyme analysis and sequencing.

Antibodies

mGluR1a constructs were detected using a mixed monoclonal antibody directed against the extracellular GFP epitope (1:150 dilution; which also recognizes CFP and YFP) that was purchased from Roche. CD8α constructs were identified using the mouse monoclonal antibody DK25 (1:50 dilution; Dako Corporation, Carpinteria, CA). Endogenous Homer protein was detected using a rabbit polyclonal antibody from Santa Cruz Biotechnology (1:100 dilution; sc-15321) that recognizes all family members of the long form of this protein, including splice variants. Other synaptic proteins were localized using antibodies against Synapsin I from Synaptic Systems, Gottingen, Germany (1:100 dilution; mouse monoclonal 106 001; rabbit polyclonal 106 002) and PSD-95 from Affinity Bioreagents (1:100 dilution; mouse monoclonal Catalog #:MA1-045, Clone 6G6-

1C9). Biotinylated donkey anti-mouse IgG and biotinylated goat anti-mouse IgG antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Alexa-633 or Alexa-546 conjugated goat anti-rabbit or goat anti-mouse IgG antibody and was purchased from Molecular Probes Inc. (Eugene, Oregon). Cy3 conjugated streptavidin was purchased from Vector Laboratories (Burlingame, CA; 1:10,000 dilution). Cy3 conjugated donkey anti-mouse IgG antibody was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) for CD8 staining. Dilutions for primary antibodies are indicated in parentheses. Secondary antibodies were all used at a dilution of 1:1,000.

Immunofluorescence Staining

For most experiments, living neurons were immunostained to detect the presence of expressed mGluR1a on the cell surface using an anti-GFP antibody (Roche) or monoclonal anti-CD8 (for CD8 chimeras). Cells were incubated with antibodies at 37 degrees for 5-10 minutes in warmed neurobasal media. Coverslips were rinsed briefly in PBS (phosphate buffered saline) prior to fixation with 4% Sucrose/4% Paraformaldehyde (in PBS) for 20 minutes at room temperature. Coverslips were rinsed three times using PBS for 5 minutes per wash and then blocked in media containing 0.5% Gelatin (Sigma Cat#:G-7765) in PBS for 30 minutes. This solution was used to dilute secondary antibodies, as well as to wash cells after antibody treatment. Cells processed for polarity measurements were not permeabilized prior to incubation with secondary antibodies.

Neurons transfected with GFP-tagged constructs were treated with biotinylated donkey anti-mouse secondary antibody for 1 hour followed by three washes in blocking medium for 5 minutes per wash. A Cy3 conjugated to streptavidin was then placed on cells at a dilution of 1:10,000 for 15-30 minutes, followed by three washes in blocking medium for 5 minutes each. Coverslips were rinsed in dH_20 prior to being mounted on slides using ProLong Gold Anti-Fade reagent (Molecular Probes). There was no amplification process for neurons transfected with CD8 chimeras; cells were incubated with Cy3 conjugated donkey anti-mouse secondary antibody alone prior to washing and mounting onto slides. Levels of transfected mGluR1a were compared to endogenous mGluR1a (corresponding to Type I mGluR1a-positive neurons; see Chapter 2). Neurons were transfected with pJPA5 CFP-mGluR1a(wild-type) and then fixed, permeabilized and blocked prior to being stained using the C-terminally directed mGluR1a-specific antibody (Chemicon). Average fluorescence intensities in dendrites of neurons endogenously expressing mGluR1a or those transfected with mGluR1a were determined by drawing regions (lines) along neuronal dendrites; these measurements were subtracted for antibody background levels. When compared, values for transfected neurons were approximately 1.4 fold higher than those of neurons endogenously expressing mGluR1a (data not shown).

Coverslips that were processed for synaptic co-localization analysis were permeabilized after fixation using 0.25% Triton X-100 reagent in PBS for 5 minutes, then washed three times in PBS for 5 minutes per wash prior to being placed in blocking solution for 30 minutes. A rabbit polyclonal antibody that recognizes all Homer splice variants (pan-Homer) was placed on cells for 3 hours. Neurons were washed three times using blocking solution for 5 minutes per wash. Secondary antibodies used were biotinylated goat anti-mouse and Alexa-633 conjugated goat anti-rabbit for 1 hour followed by washing as above. Finally A Cy3 conjugated to streptavidin was then placed on cells at a dilution of 1:10,000 for 15-30 minutes, followed by three washes in blocking medium for 5 minutes each. Coverslips were rinsed in dH_20 prior to being mounted on slides using ProLong Gold Anti-Fade reagent (Molecular Probes).

As a control, both Homer and PSD-95 were (separately) assessed for synaptic co-localization with Synapsin I in hippocampal cultures. A mouse monoclonal anti-Synapsin I was used during Homer staining whereas a rabbit polyclonal anti-Synapsin I antibody was applied during PSD-95 staining. Cells were stained using the procedure outlined above. An Alexa-633 conjugated goat anti-rabbit or goat anti-mouse secondary antibody was used to detect Synapsin I staining. Alexa-546 conjugated goat anti rabbit or goat anti-mouse secondary antibody was used for either Homer or PSD-95 staining, respectively.

Microscopy

A wide-field Leica microscope (DMRXA) microscope equipped with a 40x objective (Numerical Aperture = 0.75) was used to acquire all images used for polarity and clustering analysis. A 63x objective (Numerical Aperture = 1.32) was used to acquire images for synaptic co-localization analysis. Images were acquired using a Roper Scientific Micromax 5MHz-1300Y Camera equipped with a Sony Chip ICX061 (1300 x 1030 Interline CCD camera) controlled by MetaMorph image acquisition and analysis software (Universal Imaging Company, Downingtown, PA).

Polarity Analysis

Quantitation of the degree to which expressed constructs were polarized to dendrites was measured using the methods described in Sampo, et al (2000). Briefly, cells to be imaged were selected using the soluble YFP fill to ensure that there was no investigator bias when selecting cells for analysis. Cells which expressed both the soluble YFP and transfected construct at very high levels were excluded from analysis, as it was noted in preliminary experiments that such cells often exhibited a less polarized distribution of the exogenously expressed wild-type protein (see also Silverman, et al 2005). Images were taken such that they included the cell body of the chosen transfected cell, all dendrites and a representative sample of the primary axon and branches thereof. This usually involved capturing between 9-18 overlapping images. Images of the

chosen cells were collected in the CFP, YFP, and cy3 channels in addition to phase contrast. Cells were included only when it was certain that labeled neurites arose from the cell in question, not another nearby cell. All images were subtracted for camera background and shading correction was performed to compensate for uneven illumination. Regions consisting of single pixel lines were separately drawn on the axons and dendrites (separately) of soluble YFP image. As a control for antibody background staining, several boxed regions were drawn over cells surrounding the untransfected neuron. All regions were then (separately) transferred to the cell surface stained images of the same cell. Using Metamorph software, the average fluorescence intensity along lines and within boxes was ascertained. Average antibody background was subtracted from both axon and dendrite measurements and the average axon fluorescence was then divided by the average dendrite fluorescence, resulting in the axon:dendrite fluorescence ratio for each cell. This axon:dendrite ratio was used as a quantitative measure of polarity for all constructs.

Analysis of Clustering

Using the Cy3-surface stained panel of images that had been corrected for camera background and uneven illumination, portions of two separate dendrites were selected from each of the same cells used for polarity analysis. Care was taken to select segments of dendrites that were not crossed by other labeled neurites.

Clusters were defined as regions along dendrites whose fluorescence intensity was two-fold greater than that of the average fluorescence intensity along the whole dendrite. Metamorph software allows the user to select features in an image using *Thresholding*. The average pixel intensity for the thresholded area of the entire dendrite was then determined and antibody background (assessed as for Polarity Analysis, above) was subtracted from the average gray value in the dendrite. This value was multiplied by two to set the lower threshold for clusters. Using all the information gathered about the dendrite as a whole, as well as the regions of the dendrite designated as clusters, two parameters were assessed: cluster area (as a percent of the whole dendrite) and cluster intensity. These parameters were chosen because they are independent of the size of individual clusters, which often ran together.

Analysis of Synaptic Co-Localization

Single plane images were acquired using a wide-field Leica microscope (DMIRBE) with a 63x objective (Numerical Aperture = 1.32). Cy3 images of the cell surface stained receptor and the Alexa-633 labeled endogenous synaptic protein Homer (see Immunofluorescence procedure above) were acquired using the appropriate combinations filter. Exposure times varied such that pixel intensities were not saturated. Two separate experiments with 5-10 cells per construct were analyzed, using two dendrites per cell. Images were thresholded such that the entire dendrite was selected. Images of mGluR1a and the synaptic marker (Homer) were then compared to one another on a pixel by pixel basis

using correlation analysis. The resulting r value was a measure of co-localization. An r value close to 1.0 indicates a strong co-localization; an r value close to 0 suggests that there is little co-localization.

Analysis of Cell Surface:Total Protein Levels

Single plane images acquired in the appropriate fluorescence channel representing cell surface receptor (Cy3) or total (CFP) protein were acquired. A boxed region was used to determine the fluorescence intensity within the soma. The box was placed above the nucleus. Dendritic segments could not be used for analysis since the CFP signal diminished a great deal as the distance from the soma increased, thus resulting in an inaccurate estimation of total protein levels. Fluorescence intensities for cell surface and total protein levels were each divided by exposure times (respectively). The adjusted cell surface intensity was then divided by the adjusted total protein values for each cell. These values were calculated for each full length and mutant mGluR1a construct used in the Polarity Analysis.

RESULTS

Exogenously expressed mGluR1a was present in hippocampal dendrites where it was synaptically clustered

In the hippocampus *in situ*, mGluR1a is expressed solely in a subpopulation of GABAergic interneurons (Baude et al., 1993; Ferraguti, et al, 2004). In hippocampal cultures, mGluR1a is endogenously expressed by about 1% of neurons (Craig, et al 1993), whose dendritic morphology is typical of GABAergic neurons (Benson, et al 1994). This receptor is highly polarized to dendrites in these inhibitory neurons (Craig, et al 1993; S.Das, unpublished observations). In the current study, we examined the distribution of exogenously expressed mGluR1a in hippocampal cultures, where pyramidal neurons account for the great majority of the cells (Benson, et al 1994). Neurons were co-transfected with mGluR1a containing an epitope tag together with soluble YFP, which fills the cell's entire dendritic and axonal arborizations. Cultures were transfected at 7-9 days *in vitro* and receptors on the cell surface were assessed by live immunostaining against an extracellular epitope 5-14 days later. The level of expression of mGluR1a in transfected cells (identified by co-expression of YFP) was comparable to that in nearby cells that expressed mGluR1a endogenously, based on immunostaining with an antibody directed against the C-terminal domain (data not shown).

When exogenously expressed in hippocampal neurons, mGluR1a was present on the surface of the cell body and dendrites (Figure 1a-b). Staining extended throughout the finest dendritic branches and was also present on spines. At higher magnification, the dendritic staining appeared punctate. Staining also extended into the proximal 10-30 μm of the axon, but did not extend beyond the initial segment. The remainder of the axon, which was seen clearly with the soluble YFP was unstained for mGluR1a. Thus the distribution of mGluR1a exogenously expressed in pyramidal neurons is comparable to that seen in the interneurons that express this protein endogenously. These results suggest that the mechanisms underlying the dendritic localization of mGluR1a are shared by both pyramidal and non-pyramidal neurons.

To determine whether the mGluR1a puncta corresponded to synaptic sites, we examined cells after 21 days in culture, when synapses are fully mature. Neurons were co-stained with antibodies against Homer and PSD-95, which mark excitatory postsynaptic sites (Rao, et al 1998; Shiraishi, et al 2003). We confirmed that both Homer and PSD-95 were indeed synaptic, based on their association with synapsin-I-positive presynaptic terminals (data not shown), consistent with previous findings (Rao, et al 1998; Shiraishi, et al 2003). As shown in Figure 1C-E, the mGluR1a puncta also stained brightly for endogenous Homer. Not all Homer puncta contained mGluR1a, but nearly every mGluR1a cluster was also positive for Homer. Similar results were seen with PSD-95 (data not shown). Thus the localization of mGluR1a is comparable to that of mGluR5,

the predominant type 1 mGluR that is endogenously expressed in pyramidal neurons (Lopez-Bendito et al., 2002).

The carboxy terminal domain of mGluR1a acts as a redundant signal for polarity

When examining the role of specific sequence motifs in polarity studies, deletion of residues assesses how necessary a motif is for polarity. In contrast, appending a sequence to a reporter protein enables one to determine if the motif is sufficient to confer polarity onto an otherwise unpolarized protein. We used both approaches to study the dendritic localization of mGluR1a. First, we expressed mGluR1a constructs with mutations in the protein interaction domains that mediate binding to Homer, Tamalin and Shank (Tu, et al 1998 and 1999; Kitano, et al 2002). Figure 2 illustrates the location of these protein interaction motifs within the cytoplasmic tail of mGluR1a and the mutant constructs used in this study. These mutations have previously been shown to disrupt interactions with the respective proteins (Tu, et al 1998 and 1999; Kitano, et al 2002). The relative levels of expression of cell surface to total protein of each of these constructs was analyzed to ensure that there were no defects in trafficking (Figure 3). There was no significant difference found between the levels of expression (cell surface:total) for any of the mutant constructs, compared to that of wild-type mGluR1a.

Figure 4 a and b illustrate the localization of mGluR1a(F1156R), which is unable to interact with Homer. This mutant receptor was highly polarized to dendrites in

a manner comparable to wild-type mGluR1a (compare with Figure 1a-b). Furthermore, mGluR1a (ΔPDZ), lacking the PDZ binding motif was also restricted to the somatodendritic domain (data not shown). Since previous results suggest that the C-terminal domain of mGluR1a contributes to its dendritic localization in the retina, we next examined whether this domain contained information necessary for its dendritic polarity in hippocampal neurons. The localization of mGluR1a(ΔCT), which lacks the entire C-terminus, is shown in Figure 4 (c and d). The mutant receptor was still robustly expressed on the somatodendritic cell surface. Faint staining was also present along many axonal branches, extending to the tips of some, but not all, axonal growth cones (Fig. 4c).

The cell surface polarity of each construct was quantified as the axon to dendrite (A:D) ratio -- the average fluorescence of the axon of each cell divided by the average fluorescence of that cell's dendrites (Sampo et al., 2003). These results are shown in Figure 5. The A:D ratio for wild-type mGluR1a was 0.11, comparable to that of a number of other dendritic proteins, including the LDL receptor, which served as a control in these experiments (Jareb and Banker, 1998; Cheng et al., 2002; Silverman, et al. 2005). Mutations to either the Homer or Tamalin interaction domains caused no significant reduction in polarity (mGluR1a(F1156R): 0.09; mGluR1a(ΔPDZ): 0.13). Truncation of the entire carboxyl terminus caused a small but significant reduction in polarity (mGluR1a(ΔCT): 0.21; $p < 2x10^{-5}$). In contrast, mutating the dendritic sorting signal of LDL-R reduces its A:D ratio to 0.69, comparable to the value for other

unpolarized proteins (Jareb and Banker, 1998; Sampo, et al 2003). Taken together, these results suggest that whereas the Homer- and Tamalin/Shank-interaction motifs are not necessary for the polarization of mGluR1a, other domains within the C-terminus may make some contribution to its dendritic localization.

Although deleting the C-terminus of the receptor only modestly affected its polarity (suggesting that other regions of the protein contribute to its polarization), this does not rule out the possibility that the carboxyl tail also contains dendritic localization information. To examine this possibility, we asked whether the carboxyl terminus of mGluR1a could redirect CD8, an unpolarized protein (Craig, et al 1995), to dendrites. We prepared chimeras consisting of the ectodomain and transmembrane domain of CD8 linked to the C-terminal cytoplasmic domain of mGluR1a. Surprisingly, when this chimeric protein was expressed in hippocampal neurons, it was highly enriched on the dendritic surface, with an A:D ratio approaching that of full-length mGluR1a (average A:D ratio = 0.11). CD8 without the C-terminus of mGluR1a had average A:D of 1.10.

These experiments indicate that the C-terminus of mGluR1a contains dendritic localization information, but in the absence of the C-terminus, other regions of the receptor are also capable of maintaining dendritic polarity to a large degree. Furthermore, the polarity of mGluR1a does not depend on the motifs that mediate the receptor's interaction with Homer or Tamalin.

The polyproline motif that binds Homer was required for mGluR1a receptor clustering at synaptic sites

Previous studies have suggested that both Homer and Tamalin may play a role in scaffolding Type 1 metabotropic glutamate receptors, since co-expression of these proteins with mGluRs enhances receptor clustering in heterologous cells (Tadokoro, et al 1999). To determine whether the clustering of mGluR1a in hippocampal neurons involved interactions with these proteins, we examined the localization of the constructs described above in cells at 21 days in vitro, when synapses are relatively mature. Figure 6 illustrates high magnification images of representative cells expressing each of these constructs following live-cell staining to detect receptor on the cell surface. Results from the quantification of cluster area and intensity are indicated to the right of each image. Wild-type mGluR1a was seen in discrete clusters along the dendrites (Figure 6a; see also Figures 1c-e). Comparison with soluble YFP image (not shown) indicated that many of these puncta were present on dendritic shafts while others were localized to the tips of dendritic spines. Very little receptor staining was observed between puncta. Truncation of the carboxy terminal domain of mGluR1a resulted in a very different pattern of staining. mGluR1a(ΔCT) adopted a more uniform distribution. Bright staining was seen all along the dendritic shafts and filled the spines entirely (Figure 6b). As a negative control, we also evaluated the localization of LDL-R(Y3A), a non-synaptic protein that is uniformly distributed on

the dendritic surface (Figure 6e). The staining pattern of mGluR1a(ΔCT) resembled that of LDL-R(Y3A).

Next we examined if mutations in specific protein interaction domains could account for the disruption in clustering seen following truncation of the C-terminus. Truncation of the carboxyl PDZ binding motif had relatively little effect. mGluR1a(ΔPDZ) was present in clusters along dendritic shafts and spines (Fig 6d), similar to mGluR1a(wild-type). In marked contrast, mutation of the polyproline motif which mediates Homer binding disrupted clustering (Figure 6c), resulting in an expression pattern similar to that of mGluR1a(ΔCT). These results demonstrate that the Homer interaction domain is critical for receptor clustering, whereas the PDZ binding motif is not required.

In order to quantify these results, we developed a computer-based algorithm to identify receptor clusters, as summarized in Figure 7. We defined a cluster as a region where the fluorescence intensity was at least twice that of the average fluorescence intensity over the entire dendrite. We then computed two measures of the extent of receptor clustering: cluster area (defined as the fraction of the dendritic surface occupied by clusters) and cluster intensity (defined as the average pixel intensity within clusters divided by the average pixel intensity of the regions between clusters). Measurements were made by an observer who did not know which construct was being evaluated.

The results of this analysis are shown in Figure 8. LDL-Rmutant(Y3A), a protein that would not be expected to form clusters, served as a control for "artifactual clustering", i.e., the extent to which a uniformly distributed protein might exhibit inhomogeneities in staining. Even with this protein, there were regions of fluorescence intensity greater than twice the average, which our algorithm identified as clusters. On average, these regions occupied about 2% of the dendritic surface and had an intensity of 2.3.

In comparison, when wild-type mGluR1a was expressed, almost 9% of the dendritic surface was occupied by clusters and their intensity averaged 4.4. mGluR1a(ΔCT) exhibited a significant reduction in both cluster area and intensity compared to mGluR1a(wild-type). The reduction in clustering observed after truncating the C-terminus was entirely accounted for by a single point mutation in the Homer interaction motif (mGluR1a(F1156R)). Cluster area and cluster intensity of mGluR1a(ΔPDZ) were not significantly different from that of the wild-type receptor.

Interactions with Homer and Tamalin have also been proposed to anchor mGluR1a at and/or target the receptor to postsynaptic sites (Ango, et al 2000; Serge, et al 2002; Kitano, et al 2003). Thus mutations that disrupt receptor clustering might also be expected to abolish co-localization with postsynaptic markers. In order to confirm this, we expressed these constructs and assessed

their co-localization with endogenous Homer. The results from these studies are shown in Figure 9 and quantified in Figure 10.

As in Figure 1c-e, the clusters of wild-type mGluR1a largely co-localized with Homer (correlation coefficient: 0.60). A similar value was very obtained when NMDA receptor subunits were expressed and their distribution compared with endogenous Homer (correlation coefficient 0.65, data not shown; see also Shiraishi, et al. 2003). Truncating the entire C-terminus or mutating the Homer interaction domain greatly reduced colocalization with endogenous Homer (correlation coefficients of 0.16 and 0.18, respectively). Deleting the PDZ interaction domain had no effect on synaptic localization (correlation coefficient: 0.60). These results demonstrate that the Homer interaction motif is necessary for the synaptic clustering of mGluR1a.

DISCUSSION

In this study we tested the role of known protein interaction domains in regulating the dendritic polarity and synaptic localization of the metabotropic glutamate receptor mGluR1a. We expressed wild-type and mutant versions of this receptor in hippocampal neurons and assessed their polarity on the cell surface and the extent to which they were clustered at synapses. Our results demonstrate that the Homer interaction motif is critical for the synaptic clustering of mGluR1a. The PDZ binding domain was not necessary for receptor clustering at synapses. Neither protein binding domain was important for localizing mGluR1a to dendrites. Finally, although deleting the C-terminus only modestly reduced dendritic polarity, this domain contained sufficient information to re-localize an otherwise unpolarized protein to dendrites.

The Homer Interaction Motif

Our findings indicate that a single point mutation (F1156R) in the carboxyl domain of mGluR1a that mediates its interaction with the Homer family of proteins was sufficient to abolish the synaptic clustering of mGluR1a. Interestingly this mutation had no effect on the localization of this receptor to dendrites. Previous studies have shown that Homer can cluster mGluR1a when the two proteins are co-expressed in heterologous cells (Tu, et al 1999; Tadokoro et al 1999; Ciruela, et al 2000; Ango, et al 2002), but ours is the first evidence

that interaction via the Homer-binding motif in mGluR1a is required for synaptic localization of the receptor. While Homer is the only protein known to interact with mGluR1a via the polyproline motif, we cannot rule out the possibility that other proteins were also affected by a point mutation in this domain. Furthermore, extrasynaptic mGluR5, another member of the Group I mGluR family, exhibits increased mobility within the plasma membrane when the Homer interaction motif is disrupted; for technical reasons the authors were unable assess the role of homer in anchoring synaptic receptors (Serge, et al 2002). Taken together, these results suggest that the Group I mGluR-Homer protein interaction may be an important mechanism for tethering the receptor to particular dendritic subdomains.

As mutating the Homer interaction sequence did not disrupt receptor polarity, as compared to wild-type mGluR1a, it suggests that this motif is not responsible for regulating the localization of mGluR1a to neuronal dendrites. Interestingly Francesconi and Duvoisin found that deleting the carboxyl terminal 128 amino acids of mGluR1a (which includes both the Homer and Tamalin interaction sequences) also did not disrupt mGluR1a's localization to dendrites in retinal neurons (Francesconi and Duvoisin, 2002). Thus our results regarding polarity are consistent with previous findings.

Others have examined Group I mGluR expression in neurons and found that its trafficking to dendrites was dependent upon co-expression of Homer in rat

cerebellar granule cells (mGluR5) and cortical cultures (mGluR1a; Ango, et al 2000 and 2002; Ciruela, et al 2000). Without homer, mGluR1a (or mGluR5) was retained in the cell body. Our results do not support those findings as we did not detect any reduction in expression of mGluR1a on the cell surface with mutation that block homer interaction. Thus an interaction with Homer is not a prerequisite for efficient cell surface expression of mGluR1a in hippocampal neurons.

The Role of the PDZ Binding Domain

Deletion of the carboxyl terminal residues comprising the PDZ binding domain of mGluR1a did not disrupt either the dendritic localization or the synaptic clustering of the receptor. This result is surprising, given that PDZ interactions have previously been shown to be important for governing the polarity and synaptic clustering of a wide variety of proteins (Tejedor, et al 1997; Zito, et al 1997; Kaech, et al 1998; Rongo, et al 1998). Again, we cannot rule out the possibility that deletion of the PDZ binding motif of mGluR1a affects other proteins, in addition to Tamalin and Shank. However to date, additional proteins that interact with mGluR1a through its PDZ binding motif have not been identified.

When interpreting our results, it is important to be aware of the fact that while mutating the PDZ-interacting abolishes the direct interaction between mGluR1a and Shank (which can still bind mGluR1a indirectly via Homer), it only reduces the interaction between mGluR1a and Tamalin. Thus deleting the PDZ binding

domain of mGluR1a does not eliminate the interaction with Tamalin as other sites within the C-terminus also play a role (Kitano, et al 2002; Hirose, et al 2004). Thus in the current study, the mGluR1a(ΔPDZ) mutant construct examined likely has residual Tamalin binding but no Shank binding. However, there were no differences in clustering between mGluR1a(ΔCT), which totally lacks both Homer, Tamalin and Shank binding, and the Homer mutant -- mGluR1a(F1156R) -- which has normal Tamalin binding. Therefore it is unlikely that Tamalin binding contributes significantly to synaptic clustering. Additionally, although the disruption in clustering mediated by mGluR1a(F1156R) may also stem from an indirect interaction with Shank, we attribute Homer as the primary effector as that scaffolding protein has been characterized as the primary interacting partner at the poly-proline sequence.

Tamalin has been proposed to be important for trafficking Group I mGluRs to dendrites of hippocampal neurons (Kitano, et al 2002). However, since our results indicate that deleting the entire C-terminus (which abolishes Tamalin binding) does not reduce the receptor's ability to reach the dendritic plasma membrane, Tamalin does not appear to play a significant role in mGluR1a trafficking in hippocampal neurons.

The Role of the other regions of the C-terminus in localization of mGluR1a

We found that deleting the carboxy terminal domain of mGluR1a resulted in the appearance of some receptor on the axonal surface, but the reduction in

dendritic polarity was comparatively modest. Could it be that our approach underestimated the requirement for dendritic localization information contained within the C-terminus of mGluR1a? Perhaps the expressed mGluR1a(ΔCT) construct dimerized with an endogenously expressed GPCR, whose intact C-terminal domain enabled localization of the heterodimeric complex to the dendrites. Such a result has been described following expression of mutated AMPA receptor subunits in C. elegans (Chang, et al 2005). This possibility appears to be highly unlikely in the case of mGluR1a. Hippocampal pyramidal neurons, which account for more than 90% of the neurons in the cultures used in these experiments (Benson, et al 1994), do not express mGluR1a. Moreover, mGluR1a does not heterodimerize with other Type 1 mGluRs, even with other mGluR1 splice variants (Romano, et al 1996; Robbins, et al 1999). A recent paper has suggested that mGluR1a may heterodimerize with the calcium sensing receptor (Gama, et al 2001), another class 3 GPCR. In the hippocampus, however, CaRs are localized to presynaptic terminals (Ruat, et al 1995). Thus they are not likely to contain dendritic localization information.

Previous studies of mGluR2 in hippocampal cultures and of mGluR1a in retinal cultures found that deleting the C-terminus significantly reduced dendritic polarity (Stowell and Craig, 1999; Francesconi and Duvoisin, 2002). In our hands deleting the C-terminus resulted in the appearance of low levels of mGluR1a is axons, but the overall reduction in polarity as quantified by the A:D ratio was modest. On the other hand, when the C-terminal domain was added to CD8, the

chimera was as efficiently polarized as wild-type receptor. Thus our results indicate that the C-terminus contains dendritic localization information, but that this information is redundant; information in other domains is largely sufficient for dendritic polarization of mGluR1a. In this regard, our results parallel those of Nadler and colleagues, who studied the basolateral targeting of M3 muscarinic acetylcholine receptor in epithelial cells. They found that residues within the 3rd intracellular loop were sufficient to re-target the normally apical M2 receptor to the basolateral domain, but deletion of these residues did not disrupt the M3 receptor's basolateral distribution (Nadler, et al 2001).

With regard to synaptic clustering, it is unlikely that C-terminal motifs other than the Homer interaction domain play a significant role. Deleting the entire C-terminus did not result in any additional reduction in clustering compared with mutating only the Homer interaction domain.

Different mechanisms mediate polarity and clustering

We have demonstrated that mutations that disrupt Homer binding and abolish the synaptic localization of mGluR1a do not reduce its dendritic polarity. Conversely, Cheng et al. (2002) demonstrated that mutations that disrupt the polarity of EAAT3, a dendritically polarized glutamate transporter, do not interfere with its clustering in the dendritic membrane. Taken together, these findings suggest that distinct mechanisms underlie dendritic localization versus targeting to discrete subdomains within the dendritic membrane. Dendritic localization

likely involves selective trafficking along the biosynthetic and endocytic recycling pathways. Subcellular localization may utilize protein-protein interactions that occur after the receptor reaches the dendritic membrane. It is likely that Homer mutations interfere with anchoring, thus increasing the mobility of mGluR1a within the dendritic plasma membrane. Despite this, the diffusion barrier at the axon hillock is likely sufficient to prevent mGluR1a from reaching the axon.

Figure 1. mGluR1a expressed in hippocampal neurons was restricted to the dendritic surface and colocalized with a postsynaptic marker. (a,b) When hippocampal neurons were co-transfected with mGluR1a and soluble YFP, the YFP was present throughout the cell (**a**), whereas mGluR1a was expressed on the surface of the soma and dendrites, but not the axon (**b**). The contrast was inverted in the fluorescent image of the cell surface stained receptor to better visualize thin axonal processes. Cell bodies of nearby untransfected neurons were faintly visible, a measure of antibody background. This cell was transfected on day 9, then stained for cell surface receptor 5 days later. Arrows indicate dendrites, and arrowheads the axon. Scale bar: 20 μm. **(c-e)** To assess the synaptic localization of mGluR1a, cells were double-stained for cell surface mGluR1a and endogenous Homer after 21 days in culture. The receptor (red) appeared in distinct puncta along the surface of the dendrites **(c)**, similar to the staining pattern for Homer (green, **d**). Overlay of the two images (**e**) indicated that nearly every mGluR1a cluster co-localized with Homer (yellow). Scale bar: 5 μm.

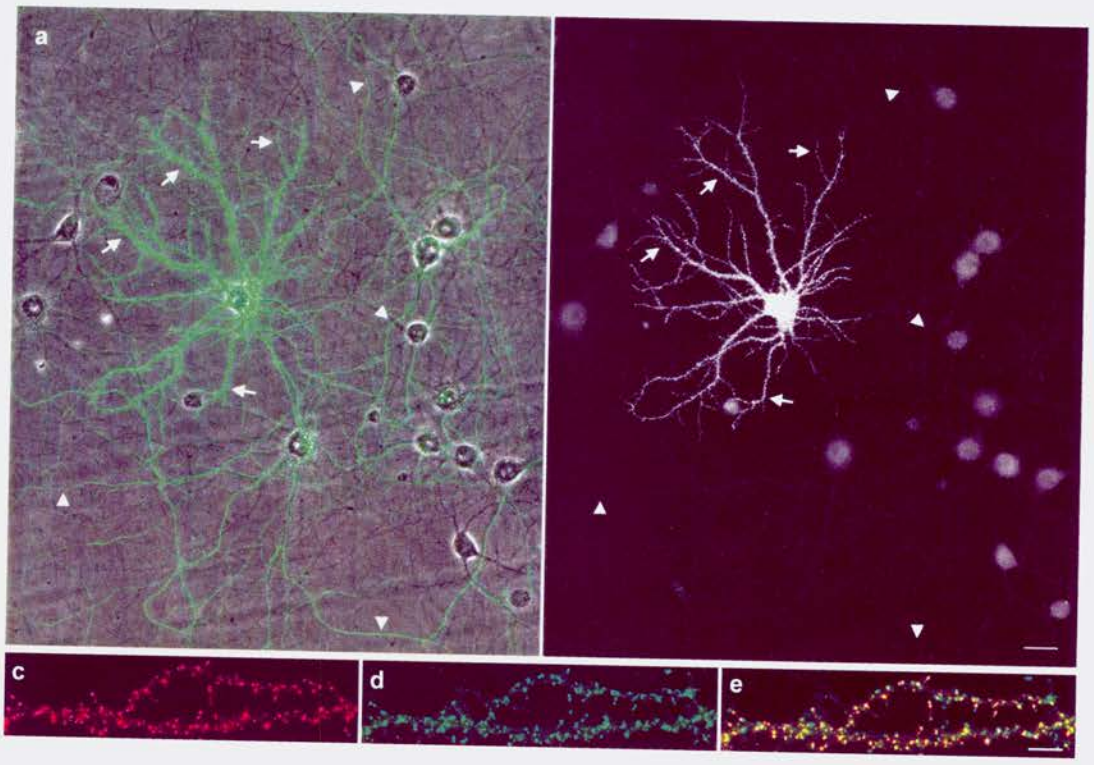
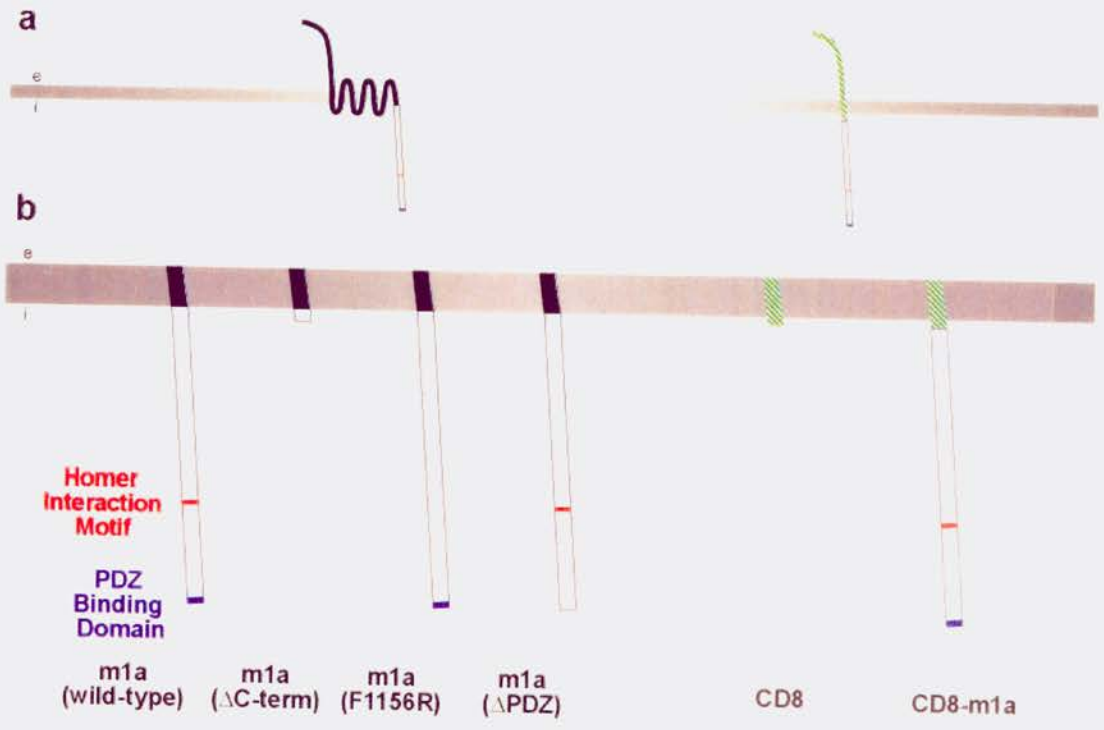

a
c
d
e

Figure 2. Mutant mGluR1a constructs used in experiments. (a) Secondary structure of mGluR1a (left) reveals a topology consisting of 7 transmembrane domains, a hallmark of G-protein coupled receptors. The topology of CD8 is presented on the right. **(b)** Schematic representation (not to scale) of the wild-type and mutant constructs used in all experiments. Mutations were introduced in the carboxy terminus only. Indicated are the 7^{th} tm (black) and carboxy terminus of wild-type mGluR1a with known protein interaction domains. The Homer Interaction Motif is indicated by the red line; the PDZ Binding Domain by the blue bar. The 3 mutant mGluR1a constructs used in experiments are illustrated (left): mGluR1a(ΔCT) was truncated 8 amino acids following the 7^{th} transmembrane domain. mGluR1a(F1156R) contains a mutation which will has been demonstrated to abolish the ability of Homer proteins to interact with mGluR1a. mGluR1a(ΔPDZ) lacking the final 3 amino acids of the receptor has previously been shown to disrupt this receptor's interaction with the protein Tamalin. Mutations or deletions of the appropriate amino acids are indicated. (right) The C-terminus of wild-type mGluR1a was introduced onto the carboxyl domain of CD8. CD8 without any additions of mGluR1a C-termini is shown.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Binding
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(wild-type)
m1a
(ΔC-term)
m1a
(F1156R)
m1a
(ΔPDZ)
CD8
CD8-m1a

Figure 3. Cell surface:total fluorescence ratios. In order to ensure that wild-type and mutant constructs were trafficked similarly to the cell surface, the cell surface immunofluoresence of each construct was compared to the total fluorescence in the same region of a neuron. Cy3 staining (representing mGluR1a construct) indicated immunofluorescence on the plasma membrane whereas CFP fluorescence indicated the total fluorescence (cell surface and intracellular).

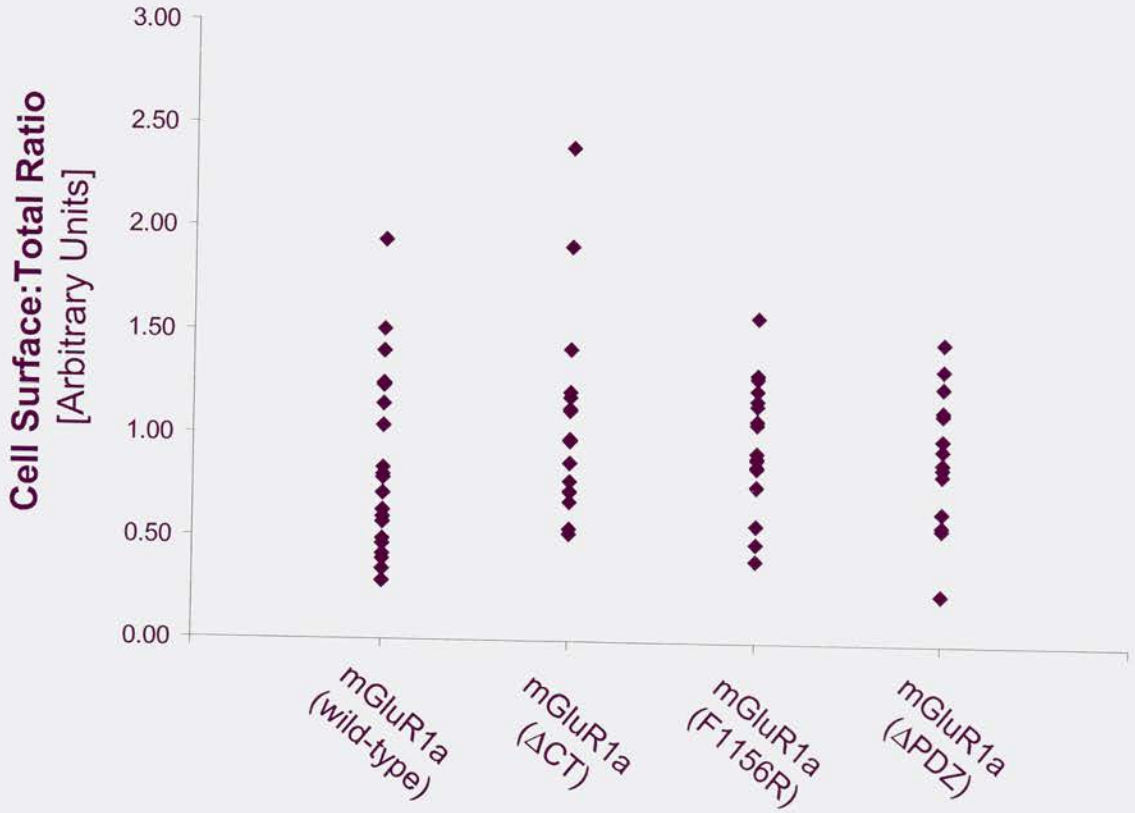

Cell Surface:Total Ratio
[Arbitrary Units]
3.00
2.50
2.00
1.50
1.00
0.50
0.00
mGluR1a (wild-type)
mGluR1a (ΔCT)
mGluR1a (F1156R)
mGluR1a (ΔPDZ)

Figure 4. The carboxy terminus of mGluR1a was largely unimportant for dendritic polarization of the receptor. In order to assess the role of the carboxy terminal domain in receptor polarity, hippocampal neurons were co-transfected (separately) with wild-type **(a-b)** or mutant mGluR1a(ΔCT) **(c-d)** and soluble YFP. As in Figure 1, the YFP filled the entire cell **(a,c)**, enabling visualization of neuronal morphology. **(b)** mGluR1a(F1156R) was similarly polarized to the somatodendritic domain as was the wild-type receptor (Figure 1a-b) **(d)** When the carboxy terminal domain of the receptor is removed, a hippocampal neuron transfected with mGluR1a(ΔCT) exhibits bright staining on the soma and dendrites as well as faint, intermittent staining along the axon. As a result of antibody background staining, cell bodies of nearby untransfected neurons are visible. Cells were transfected between 7-9 days in vitro, then stained for cell surface receptor between 5-7 days later. The contrast was inverted for all fluorescent images to enable better visualization of thin axonal processes. Arrows indicate dendrites, and arrowheads the axon. Scale bar: 20 μm.

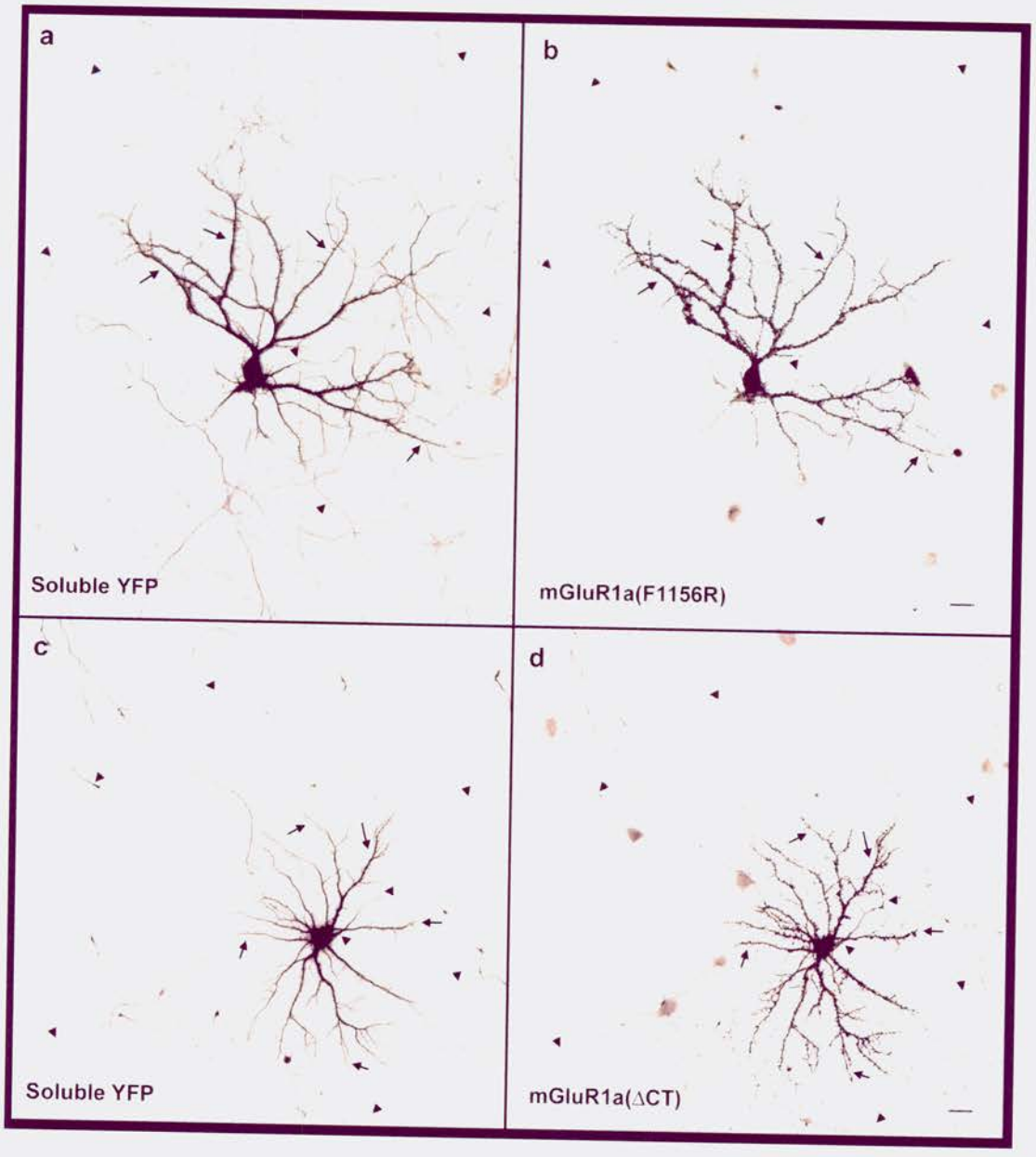

a
Soluble YFP
b
mGluR1a(F1156R)
c
Soluble YFP
d
mGluR1a(ΔCT)

Figure 5. Distribution of Axon:Dendrite Polarity Indices for all constructs. The Axon:Dendrite Ratio, a quantitative index of membrane protein polarity, was quantified for each mGluR1a construct indicated. As a comparison, the A:D ratio of another protein, the wild-type Low Density Lipoprotein Receptor (LDL-R) known to be polarized to dendrites (Jareb and Banker, 1998) was also assayed. Noticeably, mutations in mGluR1a that disrupt its ability to interact with specific proteins, do not significantly disturb its polarity in dendrites whereas truncation of the entire C-terminus does result in a significant difference in the polarity of mGluR1a(ΔCT) compared to that of mGluR1a(wild-type). Mutation of the sequences responsible for the dendritic localization of LDL-R result in a dramatic and significant (green asterisks) shift in polarity for the LDL-Rmutant(Y3A). Furthermore, this range is similar to that of the unpolarized CD8 molecule. Appending the C-terminus of wild-type mGluR1a onto CD8 results in a significant shift in polarity for this chimeric protein (red asterisks). Each data set is represented in box plot form above. The upper and lower bars represent the maximum and minimum values of the data set. The box indicates the upper and lower quartiles around a median (bar within box). Four separate experiments were done to acquire the data for the mGluR1a constructs resulting in the following values for n: mGluR1a(wild-type), n=22; mGluR1a(ΔCT), n=16; mGluR1a(F1156R), n=18; mGluR1a(ΔPDZ), n=16. Two separate experiments were done to acquire the data for the LDL-R constructs and CD8 chimaeras resulting in the following values for n: LDL-R(wild-type), n=10; LDL-Rmutant(3YA), n= 11; CD8, n=7; CD-mGluR1a(wild-type), n=6.

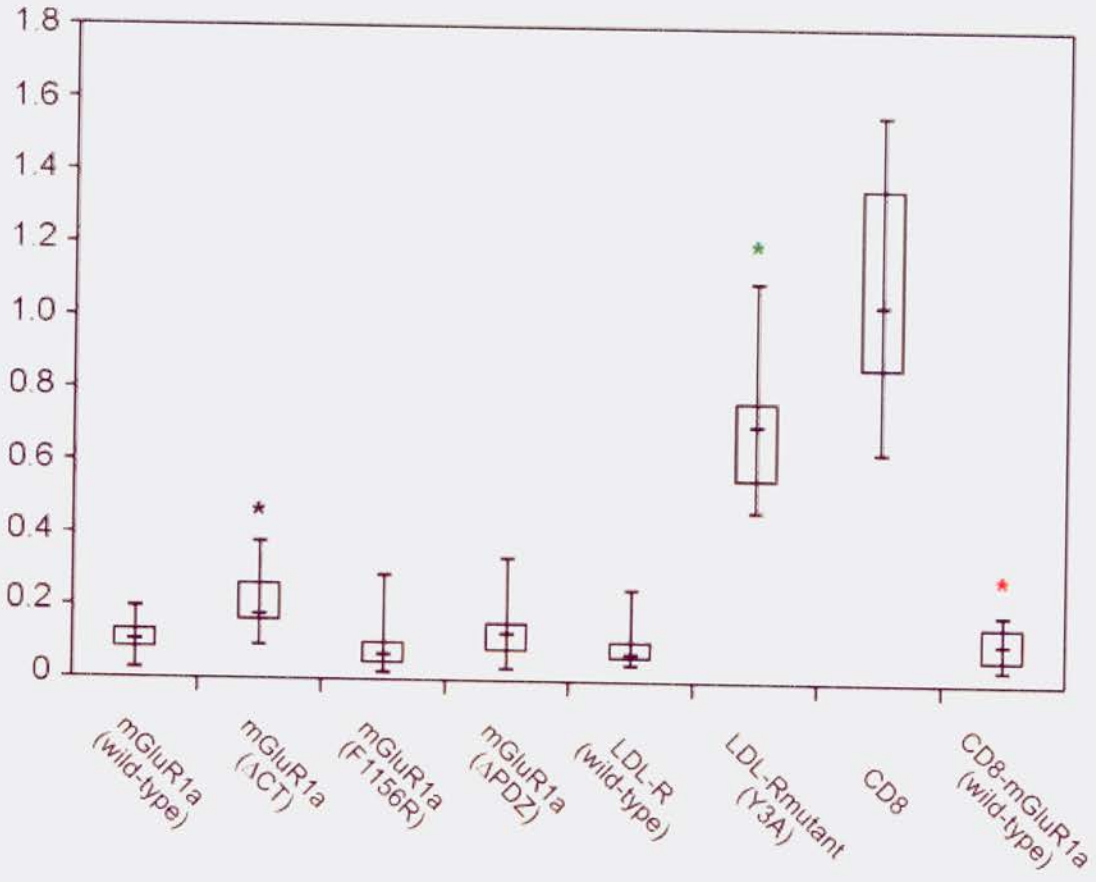

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
mGluR1a (wild-type)
mGluR1a (ΔCT)
mGluR1a (F1156R)
mGluR1a (ΔPDZ)
LDL-R (wild-type)
LDL-Rmutant (Y3A)
CD8
CD8-mGluR1a (wild-type)

Figure 6. The Homer interaction motif was required for mGluR1a clustering. Staining of neurons transfected with different constructs reveal variable degrees of cell surface clustering. Hippocampal neurons were co-transfected with wild-type or (separately) mutant mGluR1a and subsequently immunolabeled for cell surface receptors. **(a)** mGluR1a(wild-type) appears to be present on the cell surface in discrete puncta which are apparent along the shaft of dendrites and in tips of spines. **(b)** When the C-terminus of the receptor is deleted – mGluR1a(ΔCT), the receptor adopts a more uniform distribution in dendrites such that even the filipodial spines emanating from dendrites are labeled. This distribution is also observed when a single point mutation in the Homer interaction motif -- mGluR1a(F1156R) -- is expressed **(c)**. **(d)** Truncation of 3 amino acids in the carboxy terminal domain, mGluR1a(ΔPDZ), had no deleterious effect on clustering. **(e)** The LDL-Rmutant(Y3A) serves as an example of a membrane protein that is not clustered on the neuronal cell surface. Cells were transfected between 7-9 div, then stained for cell surface receptor between 5-7 days later. Scale bar: 5 μm.

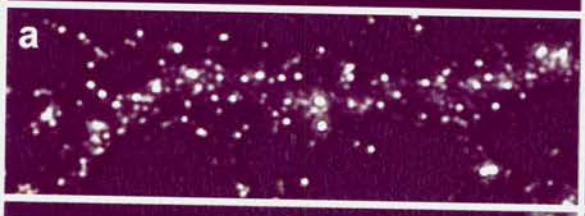
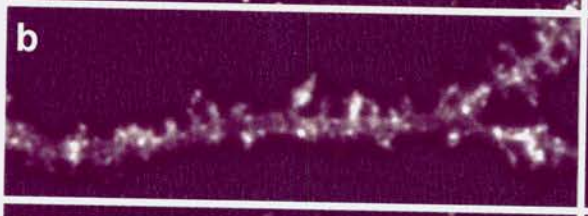
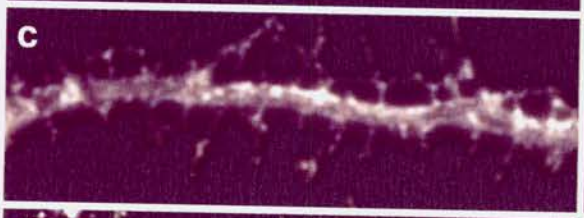
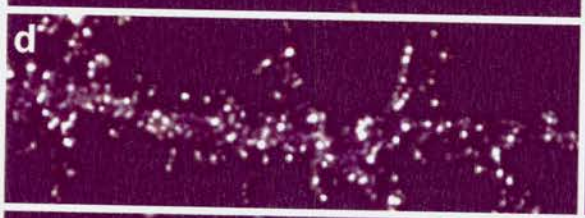
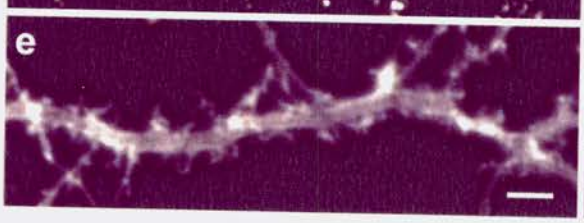
IMAGE	CLUSTER	
	Area	Intensity
 a	9.49	4.66
 b	4.89	2.63
 c	5.20	2.54
 d	9.84	3.81
 e	2.12	2.40

Figure 7. Identifying clusters. On the left is the *Original Image* of a portion of dendrite from a cell transfected with either mGluR1a(wild-type) or mGluR1a(ΔCT). The middle panel illustrates those puncta identified by the algorithm as more than twice the average fluorescence intensity of the dendrite as a whole for each construct (*Thresholded Image*). Two parameters were used as a measure of the degree of receptor clustering: the area occupied by clusters (as a percentage of the total dendritic area) and average cluster intensity. Both parameters were assessed for the images presented above for each construct.

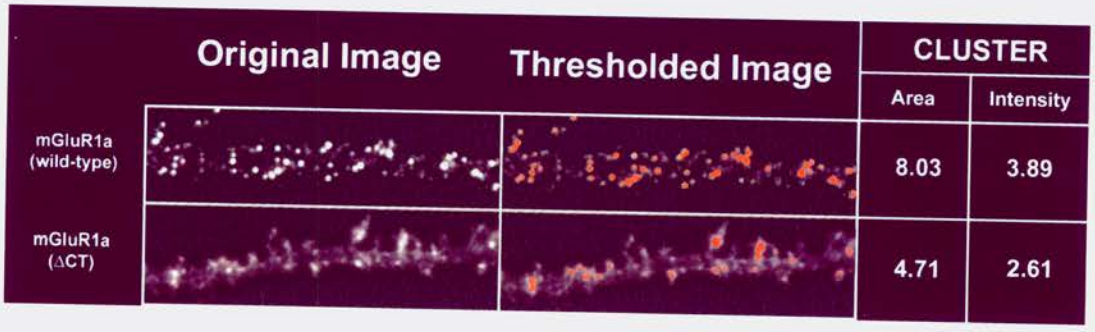

Original Image
Thresholded Image
CLUSTER
Area
Intensity
mGluR1a (wild-type)
8.03
3.89
mGluR1a (ΔCT)
4.71
2.61

Figure 8. Cluster intensity and area are dependent upon the Homer interaction motif. Cluster area results **(a)** and cluster intensity values **(b)** are graphed for each construct indicated. While mGluR1a(wild-type and mGluR1a(ΔPDZ) had similar values in both cases, the decrease in cluster area and intensity observed in the mGluR1a(ΔCT) construct could be accounted for by a single point mutation in the Homer interaction motif – mGluR1a(F1156R). LDL-Rmutant(Y3A) is present to indicate a lack of clustering, and serves as a control.

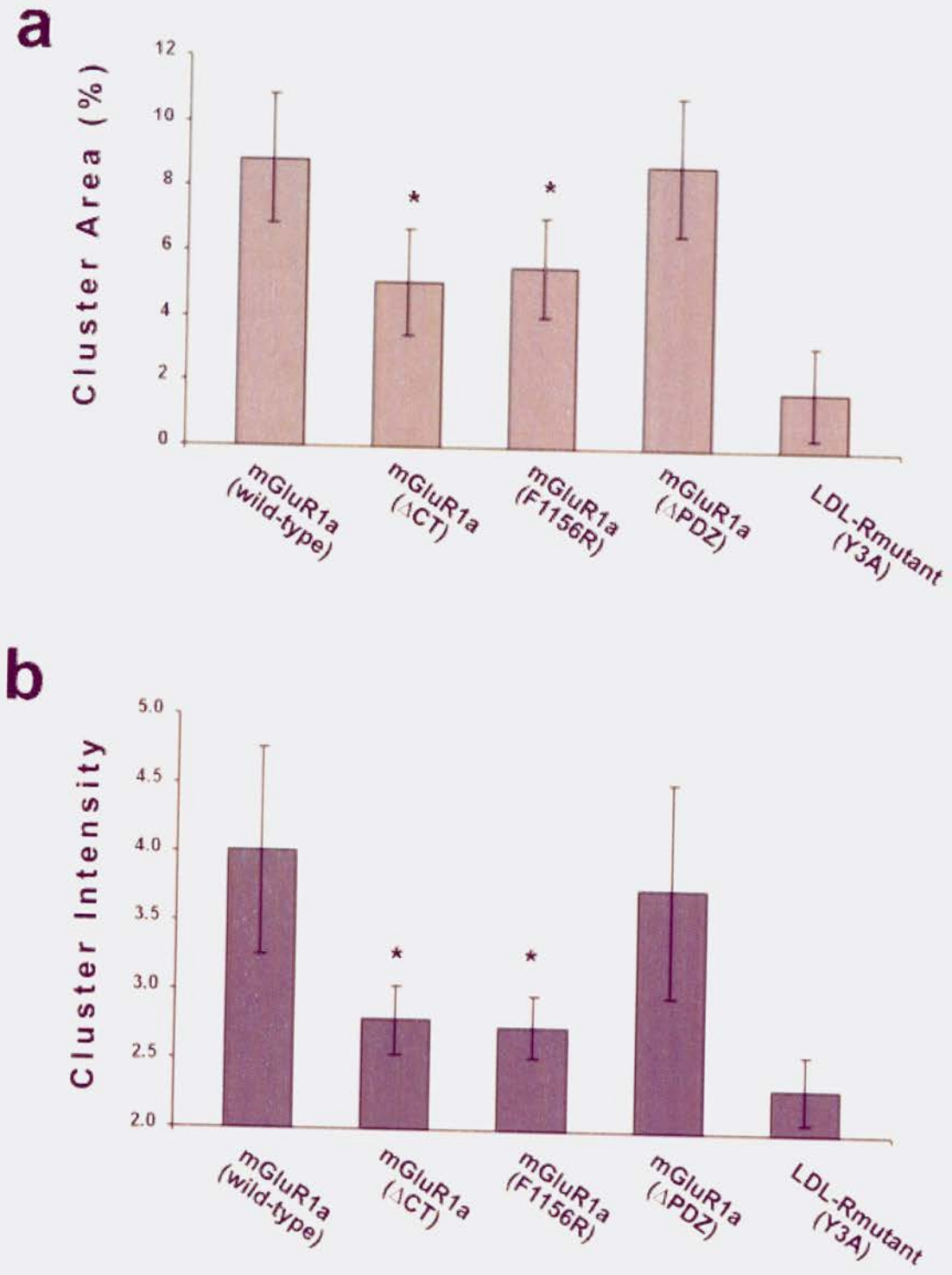

a
Cluster Area (%)
12
10
8
6
4
2
0
*
*
mGluR1a (wild-type)
mGluR1a (ΔCT)
mGluR1a (F1156R)
mGluR1a (ΔPDZ)
LDL-Rmutant (Y3A)
b
Cluster Intensity
5.0
4.5
4.0
3.5
3.0
2.5
2.0
*
*
mGluR1a (wild-type)
mGluR1a (ΔCT)
mGluR1a (F1156R)
mGluR1a (ΔPDZ)
LDL-Rmutant (Y3A)

Figure 9. The Homer interaction motif is also required for the synaptic localization of mGluR1a clusters. To determine whether the carboxy terminus, or any of the specific protein interaction domains played a role In synaptic localization, hippocampal neurons were transfected with wild-type or mutant mGluR1a. Cell were stained for cell surface receptors (red) and endogenous Homer, a synaptic protein (green). Whereas wild-type mGluR1a co-localized well with Homer, deletion of the C-terminus [mGluR1a(ΔCT)] had the same effect as a single point mutation [mGluR1a(F1156R)] which disrupts the receptor's interaction with Homer – that is mGluR1a no longer co-localized with endogenous Homer. In contrast, deletion of the domain that mediates mGluR1a's interaction with Tamalin and Shank [mGluR1a(ΔPDZ)] did not abolish the receptor's ability to co-localize with endogenous Homer, similar to what was observed with mGluR1a(wild-type). Cells were transfected 9 days in culture, then immunolabeled for cell surface receptors and endogenous Homer 12 days later. Scale bar: 5 μm.

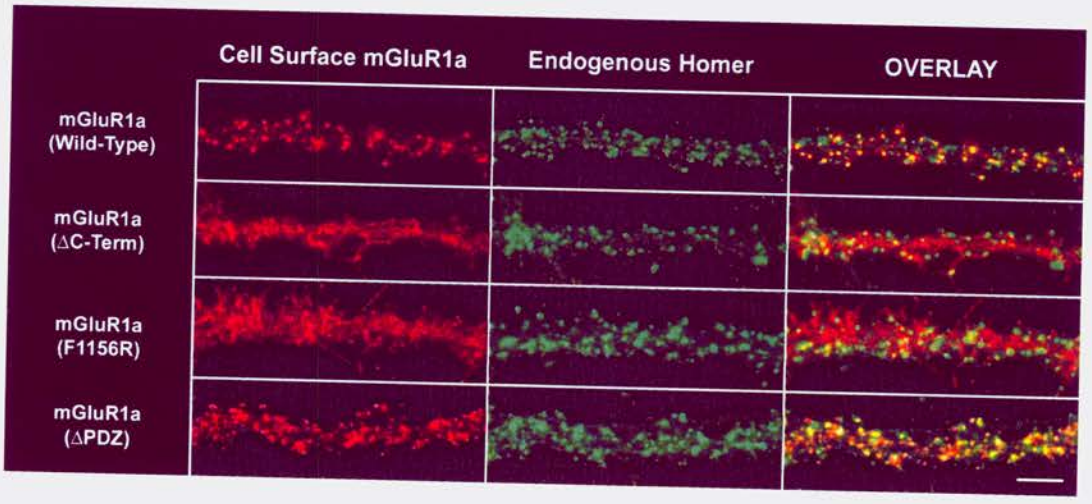
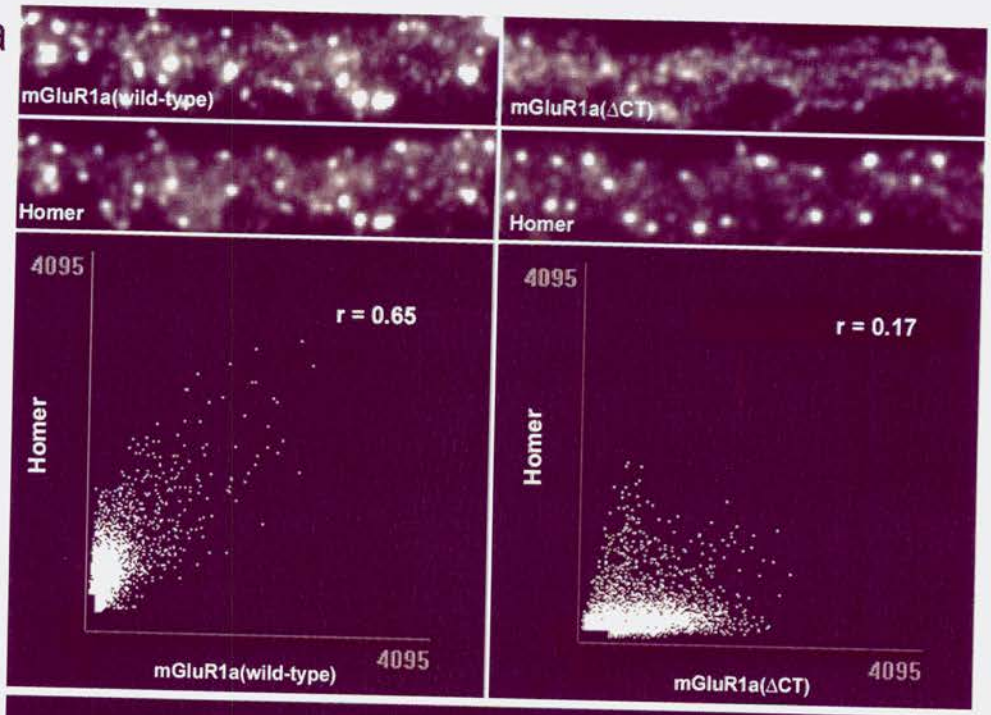
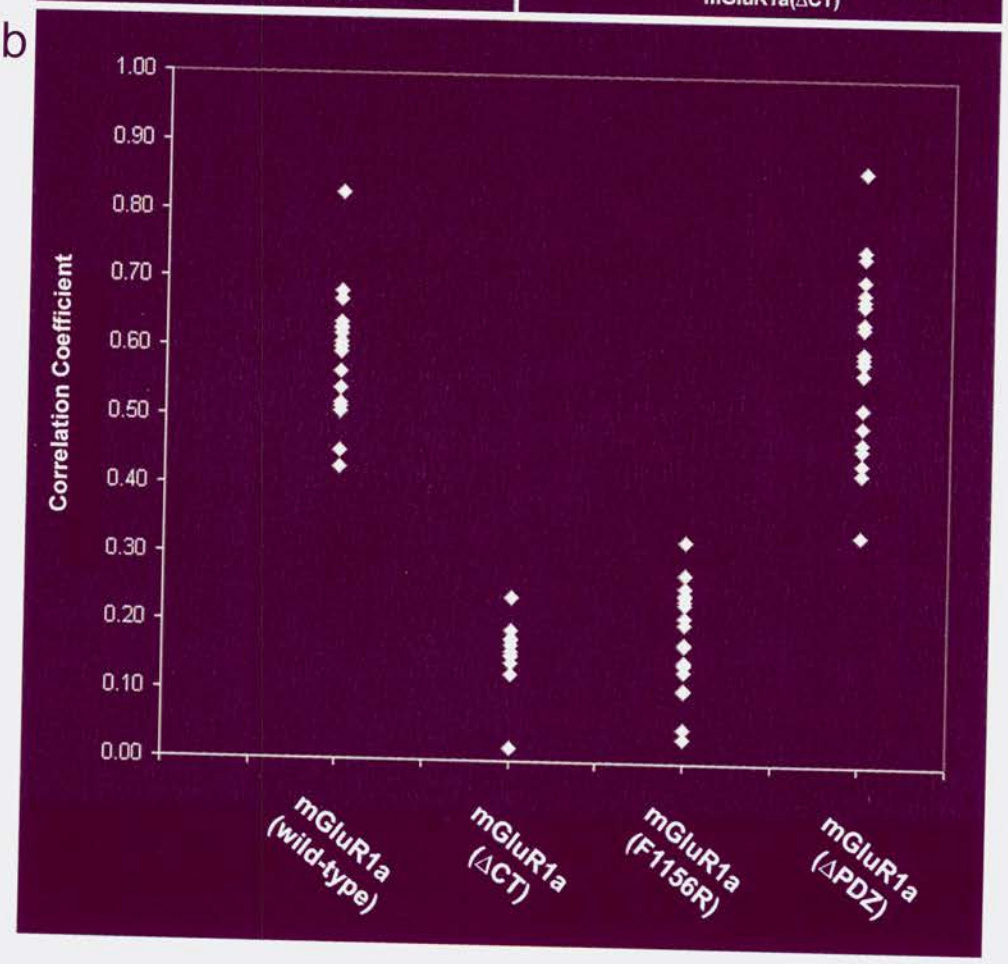

Cell Surface mGluR1a
Endogenous Homer
OVERLAY
mGluR1a (Wild-Type)
mGluR1a (ΔC-Term)
mGluR1a (F1156R)
mGluR1a (ΔPDZ)

Figure 10. Quantifying synaptic colocalization. In order to quantitate the extent to which mGluR1a wild-type and mutant receptors co-localized with endogenous Homer, the intensity of mGluR1a staining (x-axis) was compared to that of Homer staining (y-axis) on a pixel by pixel basis between images of the same dendritic segments. A correlation coefficient (r) was calculated using Metamorph software. **(a)** Shown are sample dendrites of the most extreme mGluR1a constructs, mGluR1a(wild-type) and mGluR1a(ΔCT) which either exhibit or do not exhibiting synaptic co-localization, respectively. Below the images are graphic representations of the degree of colocalization and the corresponding correlation coefficients. **(b)** The whisker plot of all correlation coefficients for each construct analyzed indicates that while mGluR1a(wild-type) demonstrated a highly significant overlap (mean r = 0. 60). Truncating the C-terminal or mutating the Homer-interaction motif greatly reduced the r value. A three amino acid truncation at the C-terminus [mGluR1a(ΔPDZ)] exhibited a synaptic co-localization akin to that of mGluR1a(wild-type).

9a


mGluR1a(wild-type)
mGluR1a(ΔCT)
Homer
Homer
4095
r = 0.65
Homer
mGluR1a(wild-type)
4095
4095
r = 0.17
Homer
mGluR1a(ΔCT)
4095

9b


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Correlation Coefficient
mGluR1a (wild-type)
mGluR1a (ΔCT)
mGluR1a (F1156R)
mGluR1a (ΔPDZ)

CHAPTER 4

SUMMARY AND CONCLUSIONS

In this dissertation, I have described several novel findings regarding the expression and trafficking of mGluR1a. I provided evidence for the existence of more than one subtype of mGluR1a-positive neurons in dissociated culture, which can be readily identified based on morphology and underlying molecular composition. I have also demonstrated the role of protein interaction domains in mGluR1a. While the Homer sequence was critical for synaptic clustering, the PDZ-binding motif was not. Although neither domain regulated the polarity of this receptor, the carboxyl terminus acted as a redundant signal to correctly localize mGluR1a to the somatodendritic domain.

Since the Homer interaction motif affected mGluR1a clustering at the plasma membrane and not its dendritic localization, it suggests that Homer does not act early in the biosynthetic pathway, in terms of sorting of mGluR1a from the endoplasmic reticulum (ER) or Golgi-apparatus. Moreover, receptor biosynthesis is not governed by Shank. Nor – in contradiction to the findings of Kitano, et al (2002) – is Tamalin necessary for trafficking of mGluR1a to dendrites. This is supported by the observation that two constructs – both mGluR1a(ΔPDZ) and mGluR1a(ΔCT) – were capable of reaching the dendritic plasma membrane equally well.

I will now discuss some possible avenues of research that follow from my work. I will also include some thoughts relevant to broader issues regarding mGluR1a and polarity.

Endogenous localization of mGluR1a

Regarding the endogenous localization of mGluR1a, it would be worthwhile to characterize the population of mGluR1a-positive interneurons in our hippocampal cultures, based on additional molecular identifiers. In addition to somatostatin, neurons known to express mGluR1a have also been found to co-express other proteins, including vasoactive intestinal peptide, calretinin and parvalbumin (Ferraguti, et al 2004). Thus although Type I mGluR1a-positive neurons do not stain for somatostatin, perhaps they express other proteins. Additionally, a further examination of Type II mGluR1a-positive cells is warranted. The patches present along dendrites are intriguing as they are not present in Type I cells. Determining whether they co-localize with pre and/or postsynaptic markers (by immunostaining for such markers) would provide useful information as to whether they represent synaptic receptor.

Finding the dendritic localization signal

An unresolved question regarding these results is "what is the dendritic localization sequence in mGluR1a?" Although I have determined that the carboxy terminal domain contains information sufficient to localize CD8 to dendrites, I did not attempt to narrow down this signal to a discrete sequence of amino acids. In order to do so, one could begin with the CD8-mGluR1a(wild-type) chimera and sequentially delete portions of the C-terminus of mGluR1a, using the Axon:Dendrite ratio to analyze the effects. Once I had found a domain

that, when deleted, resulted in a polarity similar to that of the unpolarized CD8 molecule, I would add this domain (alone) back onto both CD8 and the mGluR1a(ΔCT) to determine if this region indeed served as a dendritic localization signal for CD8, and further, as the redundant dendritic localization signal for full length mGluR1a.

My results indicate that in addition to the C-terminus, information important for the dendritic localization of mGluR1a is also present in other regions of the receptor. It is equally important to identify these domains. This would be a more challenging task. It is not obvious that one could focus on a single domain since tarageting motifs have been found throughout GPCRs, including in the C-terminus (Beau, et al 1998 and 2004; Stowell and Craig, 1999; Francesconi and Duvoisin, 2001), the 3rd intracellular loop (Nadler, et al 2001; Iverson, et al 2005) and within the transmembrane domains (Keefer, et al 1994; Yeaman, et al 1996; Saunder, et al 1998).

In order to address this question with mGluR1a, I would begin by making chimeric molecules between mGluR1a and another metabotropic glutamate receptor. In this case, I would chose an unpolarized molecule such as mGluR7, which has been shown in hippocampal cultures to be present on both the axonal and dendritic cell surfaces (Stowell and Craig, 1999). A chimeric approach would be preferential to deletional analysis of domains in mGluR1a (beside the C-terminus), due to the fact that when I remove a domain, I am not directly

assessing what hierarchical role (if any) the remaining domains play in determining polarity. When segments of two receptors are exchanged, one can more accurately determine if the remaining regions of either receptor have "dominant" roles in localization. In this way, I would hope to narrow down the dendritic localization signal of mGluR1a to a distinct region in this seven transmembrane receptor.

Addition of mGluR1a in the plasma membrane

Most of the experiments described in this dissertation were carried out using exogenous receptors, expressed at steady state (>24 hours of expression). It would be interesting to examine the question of where this receptor is first added to the membrane. In other words, is mGluR1a directly targeted to synaptic sites or is the receptor inserted throughout the dendritic plasma membrane, then anchored at synapses? The latter scenario would indicate that mGluR1a clustering at synaptic sites comes by interaction with anchoring proteins in spines, combined with elimination of mGluR1a elsewhere in the membrane.

Preliminary evidence at early time points after expression indicated that both mGluR1a(wild-type) and mGluR1a(ΔCT) were present largely in dendrites, where they appeared clustered, suggesting that both the wild-type and mutant receptors were targeted initially to the somatodendritic domain. This seems surprising since mGluR1a(ΔCT) is neither as polarized nor as clustered as mGluR1a(wild-type) at 24 hours after transfection. How then could this occur? It might be that

both receptors are intially inserted only in spines. Since mGluR1a(ΔCT) cannot interact with Homer, it would not be anchored there and thus would diffuse through the membrane. Alternatively perhaps mGluR1a(ΔCT) is inserted in spines and remains there as a result of an interaction via its remaining intracellular domains with an as-yet-unidentified-protein (protein X). If there were only a limited amount of protein X, as expression levels increased, its clustering at steady-state would be reduced. In both of the cases, the reduced dendritic polarity exhibited by mGluR1a(ΔCT) would be due to the lack of a redundant targeting signal in the carboxyl domain.

One could begin testing both hypotheses by doing a more careful polarity and clustering analysis at different time points (6, 9, 12, 15, 18, 24 hours). One caveat is that early time points might prove difficult when assessing mGluR1a in the axon if there is not a significant accumulation of receptor over background levels in the plasma membrane.

Another way to approach this question would be to use mGluR1a constructs (wild-type and ΔCT) tagged at their N-termini with a version of GFP that is pH sensitive. As intracellular carriers have a pH lower than the extracellular media, insertion of a tagged receptor would be indicated by a "flash" as the pH-sensitive GFP was exposed to a higher pH level. These experiments could be done in conjunction with total-internal reflection microscopy, or possibly epi-fluorescence

microscopy if one could remove existing fluorescence from tagged receptors at the plasma membrane.

Early events in mGluR1a trafficking

One of the things that motivated the direction of my thesis was an interest in understanding how the trafficking of synaptic proteins was differentially regulated from that of non-synaptic proteins. I wanted to examine the sorting of carriers containing these two groups of proteins early in the biosynthetic pathway. Unfortunately, imaging GFP-tagged mGluR1a-containing carriers proved very difficult due to the presence of significant fluorescence in the endoplasmic reticulum, which extends throughout all dendritic processes. Thus identifying bright, Golgi-derived carriers, moving above the background ER fluorescence has proved to be quite an undertaking.

Golgi-derived carriers are best imaged at early time points after expression. Further, by keeping neurons at a lower temperature (20 degree block), vesicle release from the golgi apparatus is inhibited. Thus a greater number of carriers can be visualized when the temperature is returned to 37 degrees and one can be confident that many of the carriers originate from the Golgi apparatus. Finally, using a spinning-disk confocal microscope might further enhance visualization of moving carriers above the background ER fluorescence. Using the techniques outlined above might make imaging of mGluR1a-carriers more feasible.

Although I have eliminated Homer and Shank as playing a role in the polarity of mGluR1a, I still do not know the means by which this receptor *is* properly sorted and trafficked to dendrites. How is polarity ensured early in the biosynthetic pathway? Although I would not co-express wild-type and mutant mGluR1a constructs in the same neuron due to dimerization issues, I could perform two-color live imaging of mGluR1a(wild-type) and another dendritic protein. I could then assess if these two proteins were trafficked in the same carrier. Using mGluR1a(ΔCT), I could then determine whether there was a significant difference in vesicle populations when this third dendritic protein was compared to carriers containing mGluR1a(ΔCT). Since the polarity of mGluR1a(ΔCT) is only slightly different from that of the wild-type receptor, I would not expect carrier populations of the mutant receptor to differ greatly from that of the wild-type receptor. However, once the dendritic targeting signal was found (see above) I could determine if mutation of all the sequences responsible for mGluR1a localization altered the carrier population of this mutant mGluR1a, compared to wild-type. Continued examination of this question would warrant imaging of mGluR1a(wild-type) versus other synaptic proteins (such as NMDA receptors), and versus an unpolarized protein, such as mGluR7. Finally, another avenue of research that has gone largely unexplored is the role that activity may play in the *sorting* of membrane proteins from the Golgi to the plasma membrane.

The work presented in this dissertation has enabled us to extend the previous findings of this laboratory which had shown that the Transferrin receptor

trafficked exclusively within dendrites (Burack, et al 2000). That both single and multi-transmembrane proteins, such as mGluR1a, are capable of being trafficked similarly suggests that neurons may employ common mechanism to restrict dendritic proteins within neurons. These results have further implications for neurobiology as they indicate that perhaps, non-synaptic and synaptic proteins share similar pathways early in the biosynthetic pathway.

Taken together, my results also illustrate just how complex the mechanisms utilized for protein localization during later steps in trafficking are. Thus, not only is protein localization dependent upon signals detected by the appropriate machinery in the ER and Golgi, but also on precise interactions with specific proteins at target membranes. While some of the motifs relevant for efficient localization of mGluR1a (at synaptic sites) have been elucidated, there are still many questions that need to be examined. The identification of motifs necessary for dendritic targeting and perhaps even the corresponding proteins enabling this localization will provide a wealth of information for neuronal cell biology. These findings may, in turn, provide clues pertinent to the trafficking of other receptors and channels that are critical for neuronal function.

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