cpg2 encodes a brain- and synapse-specific protein that regulates the endocytosis of glutamate receptors

by

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ABSTRACT

Synaptic plasticity is the rearrangement of neuronal connections that likely underlies learning and memory. It requires the expression of a set of genes essential for the synaptic changes that occur during plasticity. *candidate plasticity gene 2* (*cpg2*) was isolated in a screen for genes that effect synaptic plasticity. In this thesis, I analyze the regulation and function of *cpg2* in neurons. I find that *cpg2* is a splice-variant of the *syne-1* gene that is expressed only in brain regions capable of plasticity and encodes a protein specifically localized to a postsynaptic endocytic zone of excitatory synapses, often in the vicinity of clathrin-coated pits. I further show that, through its C-terminal coiled-coil motifs, CPG2 binds to the actin cytoskeleton and to endophilin B2, a member of a family of proteins involved in membrane trafficking. RNAi-mediated knock-down of CPG2 increased the number of postsynaptic clathrin-coated vesicles, some of which trafficked NMDA receptors, and disrupted the internalization of glutamate receptors. In addition, alterations in its protein levels affected dendritic spine size, supporting a role for CPG2 in regulating membrane trafficking. These data suggest that CPG2 organizes a network of proteins at the postsynaptic endocytic zone critical for glutamate receptor internalization. Due to its unique expression profile and subcellular localization, CPG2 may underlie a novel adaptation of the clathrin-mediated endocytosis pathway that enables the capacity for postsynaptic plasticity in excitatory synapses.

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Contents

Chapter 1

Introduction

Gene expression and synaptic plasticity

As early as the 1960's, it has been known that long-term memory requires the synthesis of new proteins (Flexner et al., 1963; Goelet et al., 1986). This early finding was supported by the fact that long-term potentiation (LTP), an experimental correlate of learning and memory (Bliss and Collingridge, 1993), requires both RNA and protein synthesis during a critical time window immediately following induction (Frey et al., 1993; Huang and Kandel, 1994; Huang et al., 1994; Nguyen et al., 1994). Thus, the cellular mechanisms of long-term memory appear to require a rapid genomic response that may result in the synaptic restructuring that is the physical substrate of long-term memory.

Similar genomic responses have been characterized in non-neuronal cells in which there is a rapid and transient activation of a group of genes called immediate early genes (IEGs) that result in cellular differentiation in response to growth factors. (Sheng and Greenberg, 1990) The expression of these genes occurs independently of protein synthesis and is therefore thought to be the primary genomic response to growth factor exposure. Many of these IEGs encode transcription factors, including members of the *fos* and *jun* families (Greenberg and Ziff, 1984; Kruijer et al., 1984; Muller et al., 1984). As

such, these transcription factors are thought to control the expression of downstream genes that are the effectors of cellular differentiation (Sheng and Greenberg, 1990).

As an example of functional conservation, many IEGs induced in cell lines by growth factors are also regulated by synaptic activity in neurons. c-*fos*, *fra-1*, *zif/268*, *nur/77*, *jun-B*, and c-*jun* are each rapidly and transiently induced in neurons following drug-induced seizures (Dragunow and Robertson, 1988; Morgan et al., 1987; Saffen et al., 1988; Sonnenberg et al., 1989; Watson and Milbrandt, 1989). IEGs are also induced following LTP paradigms. *Zif/268* is upregulated in the dentate gyrus following stimulation of the perforant path by the same paradigms that generate LTP, and its expression is dependent upon NMDA receptor activation (Cole et al., 1989). Furthermore, the number of IEGs expressed following electrical activity increases with the intensity of the stimulus (Worley et al., 1993). Importantly, IEGs are also induced by physiological levels of stimulation. Several IEGs are expressed in the visual cortex following exposure of dark-reared rats to light (Rosen et al., 1992). The synaptic regulation of IEG induction suggests that these genes may play a fundamental role in neuronal responses to activity, including synaptic plasticity.

The hypothesized importance of IEGs in the cellular mechanism of plasticity has lead to a number of studies aimed at isolating and characterizing novel IEGs expressed in the hippocampus, a region capable of significant plasticity and know to be important for learning and memory (Bliss and Collingridge, 1993). To isolate neural IEGs and not their downstream targets, several groups performed screens for genes induced by seizure in the rat hippocampus in the presence of protein synthesis inhibitors (Lanahan and Worley, 1998; Link et al., 1995; Qian et al., 1993). Worley and colleagues isolated 15 novel,

independent IEGs, approximately half of which encode transcription factors (Lanahan and Worley, 1998). This prevalence of transcription factors in the immediate response to massive synaptic activation suggests the importance of downstream gene expression in the induction of activity-dependent synaptic plasticity.

Other isolated IEGs encode proteins that are not transcription factors and may therefore be effector genes that directly result in the modificiations underlying plasticity. These IEGs encode proteins with diverse cellular functions, including 1) *cox-2*, a cyclooxygenase, a critical enzyme in the production of prostaglandins, prostacyclins, and thromboxanes (Kaufmann et al., 1996; Yamagata et al., 1993); 2) *Rheb*, a ras homologue that functions within the H-Ras/Raf-1 signaling pathway (Yamagata et al., 1994); and 3) β-activin, a member of the TGF-β family of secreted growth factors (Andreasson and Worley, 1995).

A subgroup of the activity-regulated IEGs encodes proteins that have the capacity to directly elicit morphological changes in stimulated neurons. *arc/arg3.1* was independently isolated by two separate IEG screens and encodes a member of the spectrin family of structural proteins (Link et al., 1995; Lyford et al., 1995). The protein is enriched in dendritic spines and binds to the actin cytoskeleton (Link et al., 1995), suggesting that its expression following activity may result in structural synaptic modifications. *narp* encodes a member of the pentraxin family of secreted lectins whose expression in COS-1 cells results in the enhancement of dendritic growth within co-cultured cortical explants (Tsui et al., 1996). Furthermore, Narp can directly induce the clustering of AMPA receptors in both non-neuronal and neuronal cells (O'Brien et al., 1999), suggesting that its expression during plasticity may regulate process outgrowth

and the formation of new synapses. Lastly, the *homer1a* gene product binds to the intracellular tail of the metabotropic glutamate receptors mGluR5 and mGluR1a (Brakeman et al., 1997). Its protein product is a member of a larger family of proteins that all contain a coiled-coil domain at the carboxy-terminus that binds to both IP_3 receptors and ryanodine receptors (Tu et al., 1998) and to actin filaments (Shiraishi et al., 1999). Homer 1a is unique in that it is the only activity-regulated protein in this family and does not contain the coiled-coil carboxy-terminus domain (Xiao et al., 1998). As a result, the activity-dependent expression of *homer1a* may result in the decoupling of metabotropic glutamate receptors from the smooth endoplasmic reticulum and its complex Ca^{2+} signaling processes and from the actin cytoskeleton of the dendritic spine (Xiao et al., 2000). Thus, *homer1a* expression during synaptic plasticity can affect both the signaling and structural properties of the stimulated neuron. Interestingly, each of these genes encodes a synaptic protein. The prevalence of synaptic proteins in IEG screens suggests that there is significant synaptic restructuring during periods of plasticity (Nedivi et al., 1999).

These findings show that IEG induction is the primary genomic response to synaptic activity whose expression likely generates the synaptic rearrangements necessary for plasticity. While there are some that encode effector proteins, many IEGs encode transcription factors that likely initiate the expression of a larger set of effector genes that can modify synaptic connections during synaptic plasticity. The previous screens for genes regulated by synaptic activity isolated strictly IEGs, and not their downstream targets, due tothe inclusion of protein synthesis inhibitors. These inhibitors prohibited the translation of the IEG transcription factors and the subsequent transcription

of their targets genes. These IEG target genes likely play a central role in synaptic plasticity. To isolate IEG target genes, Nedivi et al. (1993) performed a highly sensitive differential screen for genes that are induced by kainate-induced seizures in the rat dentate gyrus. Critically, no protein synthesis inhibitors were used, and the mRNA screened was collected from rat dentate gyrus six hours after seizure induction, favoring expression of IEG target genes. The screen isolated over three hundred independent clones, termed *candidate plasticity genes* (*cpg*'s), of which about two hundred are novel. These genes are likely targets of IEG transcription factors and, therefore, part of the genetic program that converts neuronal activity into functional synaptic modifications.

candidate plasticity gene 2 (*cpg2*) is a novel member of the *cpg* pool. The *cpg2* expression profile suggests that it may be involved in activity-dependent plasticity in both development and adults. It is regulated by sensory activity in the visual cortex. When rats are dark-adapted and then exposed to light, there is an increase in the expression of *cpg2* in the visual cortex as assayed by *in situ* hybridization (Nedivi et al., 1996). The timing of expression after induction by light is late and prolonged rather than rapid and transient, which suggests that *cpg2* expression has long-term rather than immediate effects. During development, *cpg2* expression begins postnatally in the rat cortex, increases until approximately two weeks, and then drops to a lower baseline in adult rat brains (Nedivi et al., 1996). Furthermore, *cpg2* expression remains high in the adult rat hippocampus (Nedivi et al., 1996), which is consistent with this region's critical role in learning and memory and its high capacity for plasticity in the adult (Bliss and Collingridge, 1993). These data indicate that *cpg2* is an activity-regulated gene that is expressed at the right

times and following the proper stimuli to play a role in developmental and adult activity-dependent plasticity.

The predicted structure of the CPG2 protein suggests a possible function. CPG2 is a 941 amino acid protein that contains two spectrin repeats. These repeats are approximately 100 amino acids in length and encode an alpha-helical rod-like domain that is common to the spectrin/dystrophin family of structural molecules (Koenig et al., 1988). Proteins of the spectrin/dystrophin family are known to be critical components of a submembrane protein skeleton, first characterized in erythrocytes, where it is thought to maintain the structural integrity of the cells as they pass through narrow capillaries. Spectrin is the primary component of the submembrane cytoskeleton, in which it forms long, rod-like multimeric complexes that bind actin and crosslink it to the membrane via a transmembrane protein complex (Ursitti et al., 1991). The spectrin cytoskeleton is found in nearly all eukaryotic cells and may subserve additional functions, including protein sorting and targeting (Beck and Nelson, 1996; Dubreuil et al., 2000; Hammarlund et al., 2000; Moorthy et al., 2000) and the regulation of intracellular trafficking (Lippincott-Schwartz, 1998). Thus, the spectrin skeleton is more than a simple membrane stabilizer but also is critical in establishing specialized membrane compartments and organizing protein networks.

Numerous members of the spectrin/dystrophin protein family are expressed in the central nervous system. Spectrin, dystrophin, and alpha-actinin have all been localized to the postsynaptic density of excitatory synapses (Jancsik and Hajos, 1998; Lidov et al., 1990; Wechsler and Teichberg, 1998; Wyszynski et al., 1997). Brain spectrin has been shown to bind to the C-terminal cytoplasmic domains of the NR1a, NR2A and NR2B

subunits of the NMDA receptor and crosslink them to the actin cytoskeleton. This interaction is antagonized by Ca^{2+} and by fyn-mediated NR2B phosphorylation (Wechsler and Teichberg, 1998). Alpha-actinin binds to the cytoplasmic tail of both the NR1 and NR2B subunits of the NMDA receptor and crosslinks them to the actin cytoskeleton, and this interaction can be antagonized by Ca^{2+}/calmodulin (Wyszynski et al., 1997). Interestingly, NMDA receptor activation can result in the signals that disrupt the binding of both spectrin and alpha-actinin to their respective NMDA receptor subunits (Wechsler and Teichberg, 1998; Wyszynski et al., 1997). These data show that spectrin/dystrophin family proteins play a role in the dendritic spines of excitatory synapses and may regulate their function and/or morphology during synaptic plasticity. Thus, the predicted structure of CPG2 suggests a structural role during synaptic function and plasticity, possibly including the maintenance of membrane compartments and/or the organization of a protein network similar to other members of the spectrin family.

In summary, IEG expression following synaptic activity likely induces the structural rearrangements that occur during synaptic plasticity. Although some encode effector proteins, a number of IEGs encode transcription factors that themselves induce the expression of downstream target genes. *cpg2* was isolated in a screen for those IEG target genes and was shown to be regulated by activity and to encode a structural protein. However, the role of *cpg2* in both neuronal function and synaptic plasticity is unknown.

Dendritic spines

Dendritic spines are protrusions from the main dendrite that occur at the sites of excitatory synaptic contacts throughout the brain. Due to their presence at excitatory synaptic sites, their capacity for signaling compartmentalization, and their regulation by

activity, they are thought to be a primary locus for synaptic plasticity (Nimchinsky et al., 2002). Dendritic spines typically range in volume from 0.01 to 0.8 cubic microns and exist at approximately 90% of excitatory synaptic contacts in the brain (Harris, 1999; Harris and Kater, 1994). They vary in their morphology into several roughly defined categories: 1) mushroom-shaped spines, with a bulbous head larger than the neck separating it from the dendrite; 2) stubby spines, where there is no neck separating it from the dendrite; 3) thin spines, in which the spine head is no larger than the neck in diameter (Harris et al., 1992; Harris and Kater, 1994). However, these categories are not static, and spine morphology often transforms from one category to another over the course of hours in immature neurons (Dunaevsky et al., 1999). These rapid transformations between spine morphologies are developmentally regulated and are reduced as neurons mature (Dunaevsky et al., 1999; Parnass et al., 2000).

Dendritic spines contain a number of organelles and subdomains, with the exact distribution varying according to brain region and neuron type. Some spines in the hippocampus contain smooth endoplasmic reticultum (SER), which serves as an intracellular store of Ca^{2+} that can be released due to synaptic activity (Spacek and Harris, 1997). Larger spines also occasionally contain a spine apparatus, a stack of SER membranes of unknown function (Gray and Guillery, 1963). Furthermore, polyribosomes are often localized to spines, raising the possibility of protein translation occurring within spines (Ostroff et al., 2002; Steward and Falk, 1985; Steward and Reeves, 1988). In fact, recent evidence shows that polyribosomes are tranlocated from the dendritic shaft to spines following plasticity-inducing stimuli, suggesting the need for local translation within spines during synaptic plasticity (Ostroff et al., 2002).

The most specialized and complex spine organelle is the postsynaptic density (PSD), an electron dense region directly apposed to the presynaptic terminal (Kennedy, 1993). The primary function of the PSD is the anchorage of glutamate receptors to the postsynaptic site. However, the PSD contains the proteins, including signaling molecules, adapter proteins, kinases, and adhesion molecules required for synaptic transmission and plasticity (Kennedy, 1993). Its size correlates with spine size and consistently occupies approximately 10% of the spine surface (Schikorski and Stevens, 1997). The number of glutamate receptors and docked synaptic vesicles is also proportional to the PSD and spine size, suggesting that larger spines contain stronger synapses (Nusser et al., 1998; Racca et al., 2000; Schikorski and Stevens, 1997; Takumi et al., 1999). This increase is glutamate receptor number is likely due to an increase in the number of "slot" proteins in the PSD that serve as docking sites for glutamate receptors (Shi et al., 2001). PSD-95 may be such a protein, as it binds to both NMDA receptors and, through the transmembrane protein stargazin, to AMPA receptors (Chen et al., 2000), and its overexpression results in an accumulation of AMPA receptors at synaptic sites (El-Husseini et al., 2000; Schnell et al., 2002).

Actin filaments are the primary cytoskeletal element within spines. Early electron microscopy studies showed that the cytoskeleton is filled with 8-10 nm diameter filaments that resemble actin microfilaments (Fifkova and Delay, 1982). It was subsequently shown by antibody staining that actin is highly concentrated in spines, (Matus et al., 1982) and labeling with fluorescently-tagged phalloidin, an actin-filament binding compound, showed a distinct punctuate staining pattern consistent with its localization to spines (Drenckhahn et al., 1984). Filaments within spines necks are

oriented parallel to the spine, while filaments within the spine head are more randomly oriented (Landis and Reese, 1983). These actin filaments are critical for multiple aspects of spine function (Halpain, 2000).

Recent evidence suggests that dendritic spines also contain a stable endocytic zone, in which membrane-associated clathrin undergoes repeated exchange with a cytoplasmic pool (Blanpied et al., 2002). It is segregated from and is localized lateral to the PSD and develops and persists independently of synaptic activity (Blanpied et al., 2002). This zone is thought to be, like the PSD, a fundamental dendritic spine specialization, dedicated to the endocytosis of spine proteins including, potentially, glutamate receptors.

Individual spines show a marked long-term stability, as they are known to persist for at least hours to days in brain slices and *in vivo* preparations (Grutzendler et al., 2002; Parnass et al., 2000; Trachtenberg et al., 2002). This is consistent with the fact that actin filaments within spines are highly stable, as they are resistant to actin depolymerizing compounds (Allison et al., 1998). However, in the short term, spines show a high degree of motility over the time span of seconds to minutes (Halpain, 2000). Spine motility was first observed when hippocampal neurons were transfected with a GFP β-actin fusion protein. The exogenous actin was concentrated in spines and showed rapid morphological changes when visualized with confocal microscopy (Fischer et al., 1998). Rapid spine motility has since been confirmed in brain slices (Dunaevsky et al., 1999; Parnass et al., 2000) and intact brain (Lendvai et al., 2000). These changes appear to occur at the edge of the spines, while the core of the spine remains stable (Fischer et al., 1998). Spine motility is blocked by the addition of the actin depolymerizing compound latrunculin A,

which demonstrates the need for dynamic actin for this process (Dunaevsky et al., 1999; Fischer et al., 1998). These data have raised the possibility that there is a core of stable actin filaments surrounded by a layer of dynamic actin filaments capable of rapid motility (Halpain, 2000).

While the function of short-term spine motility is unknown, there is evidence that it may play a role in synaptic plasticity. Spine motility is regulated, in part, by synaptic activity. Blockade of AMPA or NMDA receptors results in the inhibition of actin dynamics and spine protusions, resulting in smaller, rounded spines (Fischer et al., 1998; Korkotian and Segal, 2001a). When electrically stimulated, there is an increase in protrusions at the spine tip, away from the central actin core (Fischer et al., 1998). Furthermore, spines have been shown to 'twitch' during back-propagating dendritic action potentials, movements dependent upon a rise in intracellular calcium concentration (Korkotian and Segal, 2001b). It has further been shown that long-term potentiation requires dynamic actin filaments, showing that spine motility may be required for synaptic plasticity (Krucker et al., 2000). Thus, since they are the primary site of excitatory synaptic transmission in the brain and are modified by synaptic activity, dendritic spines may be a primary target for activity-regulated genes during synaptic plasticity.

Glutamate receptor trafficking in excitatory synapses

LTP is the selective enhancement of synaptic transmission by a stimulation often designed to maximize NMDA receptor activation and Ca^{++} influx (Bliss and Collingridge, 1993). Its converse, LTD, is the selective depression of synaptic strength following a specific stimulation paradigms that minimizes Ca^{++} influx (Kirkwood et al.,

1993). These experimental phenomena are thought to be cellular correlates of the synaptic plasticity mechanisms that underlie learning and memory (Bliss and Collingridge, 1993). While the mechanism for alterations in synaptic strength during plasticity is still controversial, there is ample evidence that it might be, at least in part, due to changes in the number of glutamate receptors at the synapse (Malinow and Malenka, 2002). Such a mechanism would require the selective insertion or removal of glutamate receptors depending upon the stimulation paradigm and the resulting form of plasticity. Recent data has begun to uncover the mechanisms of AMPA and NMDA receptor trafficking into and out of the synapse.

One well-established phenomenon of LTP is the "silent synapse", wherein synapses that only display NMDA receptor currents can be activated by plasticity-inducing stimuli to display AMPA receptor currents, thus making them capable of synaptic transmission at resting potential (Liao et al., 1995). While there is likely to be a presynaptic component to this phenomenon (Renger et al., 2001), it is widely believed that AMPA receptors are rapidly inserted or translocated to the synaptic site following the stimulation protocol (Malinow and Malenka, 2002). Conversely, AMPA receptor internalization is thought to be an important component of LTD (Beattie et al., 2000; Man et al., 2000; Snyder et al., 2001). Thus, the regulation of synaptic AMPA receptor trafficking is likely a critical component of synaptic plasticity.

AMPA receptors are composed of four subunits (GluR1-4) (Ozawa et al., 1998). Each of these subunits has a unique C-terminal cytoplasmic tail that interacts with a unique set of intracellular signaling and structural proteins, which underlie different mechanisms of exocytosis (Passafaro et al., 2001; Shi et al., 2001). Using an

electrophysiological tag and virally introduced AMPA receptor subunits, it has been shown that, similar to the induction of LTP, the insertion of GluR1 homomers into synapses is dependent upon synaptic activity, and specifically NMDA receptor and CaMKII activation (Shi et al., 2001). Conversely, GluR2 is inserted independently of activity and does not result in alterations of synaptic strength, suggesting that, while GluR1 insertion enhances synaptic strength by the addition of new receptors, GluR2 insertion is a steady state mechanism of maintaining synaptic strength (Shi et al., 2001). However, in GluR1-GluR2 heteromers, AMPA receptors are trafficked by the GluR1 rules, showing that the GluR1 cytoplasmic tail likely governs the behavior of endogenous heteromeric receptor exocytosis (Shi et al., 2001). Similar results have been shown using a thrombin cleavage assay in cultured hippocampal neurons (Passafaro et al., 2001). Although the mechanism underlying GluR1 containing AMPA receptor insertion during synaptic plasticity is unknown, recent evidence suggests that the small GTPase Ras relays the CaMKII and NMDA receptor activation required for GluR1 exocytosis (Zhu et al., 2002).

The regulation of AMPA receptor internalization is similarly complex. AMPA receptors are constitutively and rapidly internalized, at a rate regulated by synaptic activity (Ehlers, 2000; Lin et al., 2000). Furthermore, the internalization of AMPA receptors plays a critical role in LTD. Treatment of hippocampal neurons with agents that cause LTD, including insulin, NMDA, and a metabotropic glutamate receptor agonist, stimulate the internalization of AMPA receptors (Beattie et al., 2000; Man et al., 2000; Snyder et al., 2001). Like its exocytosis, AMPA receptor internalization has subunit specific regulation. However, the rules are reversed. While both GluR1 and GluR2 are

constitutively internalized, GluR2 shows enhanced internalization in response to AMPA and NMDA receptor activation, and the GluR2 tail dominates in heteromeric receptors (Lin et al., 2000). Recent evidence suggests that the small GTPase Rap may be an important regulator of AMPA receptor internalization (Zhu et al., 2002).

NMDA receptors have historically been thought to play a critical role in the induction but not the expression of LTP and LTD (Malinow and Malenka, 2002), resulting in less interest in understanding the NMDA receptor trafficking. However, recent evidence shows that NMDA receptors are trafficked rapidly into and out of the synapse during plasticity events, suggesting NMDA receptor trafficking may be a locus of synaptic plasticity. In the brain, NMDA receptors are composed of the NR1 subunit and a mixture of NR2A-D subunits (Ozawa et al., 1998). During development, there is a gradual shift from NR2B to NR2A subunit containing receptors, resulting in a shortening of NMDA receptor-mediated currents (Monyer et al., 1994). When animals are dark-reared, the switch to NR2A containing receptors is delayed (Quinlan et al., 1999a). However, when dark-reared animals are exposed to light, there is a rapid (<1hr) insertion of NR2A subunits, demonstrating the activity-regulation of NMDA receptor exocytosis (Quinlan et al., 1999b). Furthermore, contrary to previous plasticity studies, the use of a novel stimulating electrode to induce LTP leads to a rapid insertion of NMDA receptors into the synapse in a PKC and Src-dependent manner, suggesting that NMDA receptor trafficking may play a bigger role in the expression of plasticity than previously believed (Grosshans et al., 2002).

The exocytosis of NMDA receptors is regulated by signals involved in synaptic plasticity. Protein kinase C (PKC) controls the trafficking of NMDA receptors into the

synapse by two mechanisms with different time courses. The NR1 C-terminus cytoplasmic domain contains an ER retention sequence (Scott et al., 2001; Standley et al., 2000). When Ser896 and Ser897, immediately adjacent to this sequence, are phosphorylated by PKC in COS7 cells, there is a slow accumulation of NMDA receptors on the cell surface over the course of hours (Scott et al., 2001). Furthermore, PKC induces the rapid exocytosis of NMDA receptors into synapses of cultured hippocampal neurons not by the direct phosphorylation of the receptors, but possibly by the phosphorylation of the exocytosis protein SNAP-25 (Lan et al., 2001).

Like AMPA receptors, NMDA receptors are constitutively internalized, but at a slower rate that is not regulated by activity (Ehlers, 2000; Lin et al., 2000), and certain signals can induce their internalization. The activation of metabotropic glutamate receptors causes the rapid endocytosis of NMDA receptors (Snyder et al., 2001). Furthermore, NMDA receptor internalization rules depend upon the subunit composition of the receptor. NR2B containing receptors are rapidly internalized, while NR2A containing receptors are relatively stable in synapses (Roche et al., 2001).

Glutamate receptor internalization is thought to occur through clathrin-mediated endocytosis. Clathrin-mediated endocytosis is a general mechanism for the internalization of proteins from the plasma membrane (Mousavi et al., 2004). It is a multi-step process. First, clathrin forms a cage-like lattice at an invaginating developing pit and is accompanied by the recruitment of cargo proteins to be internalized, often by the binding of the AP2 clathrin-adapter protein complex to the internalized protein. The forming vesicle then pinches off from the membrane in a dynamin-dependent step, the clathrin-coat is removed, and the vesicle is trafficked to an endosomal compartment

(Kirchhausen, 2000). Inhibitors of clathrin-mediated endocytosis block both glutamate receptor internalization and LTD induction (Beattie et al., 2000; Carroll et al., 1999; Ehlers, 2000; Lin et al., 2000; Man et al., 2000). Furthermore, GluR2 and NR2B interact with AP2, and the endocytosis of both requires this interaction (Lavezzari et al., 2003; Lee et al., 2002). These interactions with AP2 may be critical for the recruitment of glutamate receptors to clathrin-coated pits.

Events that cause the unbinding of glutamate receptors from the PSD have also been shown induce their internalization. Phosphorylation of the GluR2 tail by PKC decreases its surface expression and prevents its association with GRIP and ABP, PSD proteins that serve to anchor AMPA receptors (Chung et al., 2000; Matsuda et al., 1999). For NMDA receptors, deletion of the NR2B binding site of NMDA receptor-docking protein PSD-95 increases the rate of NMDA receptor internalization (Roche et al., 2001). PSD-95 is also thought to act as a docking site for AMPA receptors at the synapse, through its interaction with the transmembrane, AMPA-receptor binding protein stargazin (Chen et al., 2000; Schnell et al., 2002). Recent evidence suggests that ubiquitination-dependent degradation of PSD-95 results in the internalization of AMPA receptors (Colledge et al., 2003). PSD-95 is ubiquitinated in response to NMDA receptor activation and rapidly degraded by the proteasome. Blocking PSD-95 ubiquitination decreases NMDA receptor-dependent AMPA receptor endocytosis, while inhibition of the proteasome inhibits LTD (Colledge et al., 2003). Thus, activity may regulate glutamate receptor internalization by controlling their anchorage to the PSD by the phosphorylation state of glutamate receptor tails and by enhancing the degradation of anchoring proteins such as PSD-95.

The mechanism of glutamate receptor endocytosis in neurons, and whether it is similar to endocytosis in other cell types, is unclear. Given the importance of glutamate receptor trafficking for synaptic plasticity, neurons may have developed unique mechanisms for regulating glutamate receptor endocytosis. However, the proteins and mechanisms underlying this process, and whether they differ from clathrin-mediated endocytosis in other cell types, are poorly understood. Internalization of AMPA receptors is disrupted in neurons from huntingtin interacting protein 1 (HIP1) knockout mice (Metzler et al., 2003). Since HIP1 is an endocytic protein expressed in many tissues (Kalchman et al., 1997; Wanker et al., 1997), this result may be a general defect in endocytosis and not specific to glutamate receptor trafficking.

In cell lines, clathrin-mediated endocytosis occurs at discrete and stable "clathrin-pit zones" on the membrane (Gaidarov et al., 1999; Santini et al., 2002; Scott et al., 2002). A recent study using GFP-tagged clathrin revealed a subdomain of dendritic spines, segregated from the PSD, devoted specifically to clathrin-mediated endocytosis (Blanpied et al., 2002). This zone is stable and is formed independently of activity (Blanpied et al., 2002). Post-embedding immunogold labeling showed that clathrin is concentrated in a region approximately one PSD length away from the PSD. (Racz et al., 2003) Since PSD size tightly correlates with spine size (Schikorski and Stevens, 1997), the distance of the endocytic zone from the PSD correlates with the spine diameter (Racz et al., 2003), suggesting a tight regulation of the formation of this structure. These results are consistent with other electron microscopy-based studies that have shown the presence of clathrin-coated pits and vesicles in dendritic spines, normally separated from the PSDs (Cooney et al., 2002; Petralia et al., 2003; Spacek and Harris, 1997; Toni et al., 2001).

Interestingly, the localization of the endocytic zone separated from the PSD would require the dissociation of glutamate receptors from the PSD for their internalization, fitting with the data suggesting that dissociation from the PSD induces receptor internalization. As a stable synaptic component, this endocytic zone may be a part of a postsynaptic specialization for glutamate receptor internalization.

Summary

Little is known about how the downstream targets genes of the IEGs effect the synaptic plasticity that likely underlies learning and memory. Isolated in a screen for IEG target genes, *cpg2* is a novel transcript whose expression is consistent with a role in synaptic plasticity. Its encoded protein contains cytoskeletal motifs similar to members of the spectrin family that organize protein complexes and is thus likely to play a similar role in neurons during synaptic function and plasticity. In this study, I address the expression, regulation, and function of *cpg2* in neurons. In Chapter 2, I show that *cpg2* is an activity-regulated splice-variant of the *syne-1* gene whose expression is limited to brain areas capable of synaptic plasticity, examine the activity-regulation of *cpg2* transcription and translation, and show that CPG2 is specifically localized to the postsynaptic endocytic zone of excitatory synapses. In Chapter 3, I examine potential CPG2-interacting proteins and demonstrate that CPG2 interacts with itself, the actin cytoskeleton, and endophilin B2, a member of a family of proteins involved in membrane trafficking. In Chapter 4, I address the function of CPG2 in neurons by manipulating its levels with overexpression and RNA interference-mediated knock-down and find that CPG2 is critical for the internalization of both AMPA and NMDA receptors. Chapter 5 presents a model of the function of CPG2 within dendritic spines wherein CPG2 is an organizer of a novel

modification of the clathrin-mediated endocytosis pathway controlling glutamate receptor internalization in excitatory synapses with the capacity for significant postsynaptic plasticity.

Chapter 2

cpg2 is a brain-specific, activity-regulated splice-variant of *syne-1* that encodes a postsynaptic endocytic protein

Abstract

cpg2 was isolated in a screen for transcripts regulated by activity in the rat dentate gyrus and was subsequently shown to be regulated by light in the visual cortex. In this chapter, I characterize the expression of *cpg2* and its regulation by activity and assess the activity-regulation and subcellular localization of the CPG2 protein. I find that *cpg2* is one of two closely-related splice-variants of the *syne-1* gene that are expressed only in brain regions capable of synaptic activity, and its expression is increased multiple-fold following seizure. Using a novel anti-CPG2 monoclonal antibody, I show that the CPG2 protein, which solely contains protein interaction motifs, is unaffected by seizure and is specifically localized to a postsynaptic endocytic zone of excitatory synapses. Thus, CPG2 is encoded by a brain-specific activity-regulated splice-variant of *syne-1* and is ideally situated for the organization of a protein network that regulates postsynaptic endocytosis at excitatory synapses.

Introduction

Learning and memory and synaptic plasticity both require gene transcription and protein translation for long-term expression (Flexner et al., 1963; Frey et al., 1993; Goelet et al., 1986; Huang and Kandel, 1994; Huang et al., 1994; Nguyen et al., 1994). Immediate early genes (IEGs) are the initial sets of genes that are expressed following synaptic activity (Sheng and Greenberg, 1990). Many encode transcription factors (Lanahan and Worley, 1998), suggesting that there are effector genes expressed by these IEGs that produce the synaptic changes that underlie plasticity and learning and memory (Nedivi et al., 1999). Nedivi et al. (1993) performed a screen designed specifically to isolate target effector genes that are induced by synaptic activity in the rat dentate gyrus. This screen isolated approximately 300 independent activity-regulated transcripts, termed *candidate plasticity genes* (*cpg*'s), of which about 200 are novel. Each of these transcripts encodes a protein that potentially plays a role in effecting synaptic plasticity.

cpg2 is a novel member of the *cpg* pool. Preliminary analysis has shown that it is upregulated in the rat dentate gyrus following kainic acid-induced seizure (Nedivi et al., 1993) and is expressed postnatally in the rat cerebral cortex and hippocampus (Nedivi et al., 1996). Furthermore, in dark-adapted rats, its expression is increased in the visual cortex following exposure to light. This upregulation is prolonged, lasting for over 24 hours, suggesting that *cpg2* has long-term effects on the neurons in which it is expressed (Nedivi et al., 1996). This expression pattern is suggestive of a role for *cpg2* in long-term synaptic plasticity.

The *cpg2* transcript encodes a 941 amino acid protein. This protein contains exclusively motifs that predict an intracellular structural protein from the

spectrin/dystrophin protein family (Nedivi et al., 1996). These proteins are known to regulate cytoskeletal structure and to underlie the localization of protein networks at the plasma membrane (Beck and Nelson, 1996; Dubreuil et al., 2000; Hammarlund et al., 2000; Moorthy et al., 2000). Members of this protein family, including spectrin, dystrophin, and alpha-actinin are localized to the postsynaptic site of excitatory synapses, and several have been shown to interact with glutamate receptors (Jancsik and Hajos, 1998; Lidov et al., 1990; Wechsler and Teichberg, 1998; Wyszynski et al., 1997).

Preliminary studies on *cpg2* expression left a number of remaining questions as to the expression, regulation, and localization of both the *cpg2* transcript and the CPG2 protein. In this chapter, I show that the *cpg2* transcript is one of two closely related spice-variants of the *syne-1* gene whose expression is limited to regions of the brain capable of synaptic plasticity and is increased multiple-fold in the brain following seizure induction. The CPG2 protein is translated in the brain, and its levels remain constant following seizure induction despite the multiple-fold increase in *cpg2* transcript levels. Lastly, CPG2 is specifically localized to an endocytic zone at the postsynaptic site of excitatory synapses, often in the vicinity of clathrin-coated pits. These data suggest that CPG2 may regulate the endocytosis of postsynaptic proteins specifically in synapses capable of synaptic plasticity.

Results

Analysis of *cpg2* transcription

A partial *cpg2* cDNA isolated in a screen for activity-regulated genes (Nedivi et al., 1993) was used as a probe for isolating the full-length *cpg2* clone from a rat dentate gyrus

cDNA library. The full-length cDNA contains a 2.8 kB open reading frame predicting a protein of 941 amino acids flanked by a 266 nucleotide 5' untranslated region (UTR) and 2.3 kB 3' UTR (Nedivi et al., 1996). Database searches using the *cpg2* cDNA sequence identified exons 16-33 of the *syne-1* gene as corresponding to the 18 exons of the *cpg2* coding region, with the *syne-1* exon 34 contained in the *cpg2* 3'UTR (Figure 2-1A). *syne-1* encodes a large protein with an actin-binding domain at the N-terminus and a nuclear membrane localization domain at the C-terminus, separated by a long, helical separator region (Starr and Han, 2003). The *cpg2* exons are from the region of the gene that encode the separator region of the Syne-1 protein. A Northern blot of total RNA from rat cerebral cortex probed with a segment of the *cpg2* coding sequence identified a single 5.9 kB transcript (Figure 2-1B), the approximate size of the longest *cpg2* cDNA clone.

Analysis of the *cpg2* 3'UTR reveals the likely sites of *cpg2* early transcription and translation termination. The *cpg2* 3'UTR contains an unspliced intron between exons 33 and 34 of the *syne*-1 gene. Exon 34 is followed by a non-canonical polyadenylation hexamer, conserved in rats and humans, which likely serves as the site of *cpg2* transcription termination (Figure 2-1C). The unspliced intron between exons 33 and 34 contains a stop codon 7 codons downstream of the end of exon 33, which serves as the translation termination site.

Since it was unclear whether the original *cpg2* cDNA contained the complete 5'UTR, 5'RACE was used to map the *cpg2* 5'UTR and isolated two separate bands (Figure 2-1D). The sequencing of these bands identified two 5' ends to the *cpg2* transcript. One transcript (*cpg2*) corresponds to the original *cpg2* cDNA isolated in the screen. A second, novel transcript (*cpg2b*; Accession #AY597251) contains one

additional exon at the 5' end of the *cpg2* coding region (Figure 1E). A mouse clone homologous to *cpg2b* has been isolated from an olfactory bulb cDNA library (Accession #AK032502). The *cpg2b* open reading frame codes for an additional 24 N-terminal residues that contain no known structural domains.

Since *cpg2* and *cpg2b* differ in their 5'UTR sequences, they have different transcription start sites. To examine the transcription initiation of *cpg2* and *cpg2b*, rat and human genomic sequences near the site of their transcription initiation were compared, and conserved potential TATA boxes and transcription factor binding sites were identified (Figure 2-2). Upstream of the *cpg2b* start site, there is a TATA box conserved in rats and humans that likely serves as the site of transcription initiation. There is no TATA box upstream of *cpg2*, indicating that it results from TATA-less transcription initiation. The only conserved, potential transcription factor binding sites in this region are an AP-1 site, just downstream of the *cpg2b* initiation site, and two C/EBP sites, one upstream of and one at the site of *cpg2* initiation. Both of these transcription factors are regulated by activity (Sheng and Greenberg, 1990; Yukawa et al., 1998), suggesting that they may underlie the activity-dependent transcription of these transcripts.

To determine the tissue distribution of *cpg2* and *cpg2b*, RT-PCR was performed using sense primers unique to each transcript and an antisense primer common to both (see Figure 2-1E). While *β-actin* amplified from cDNA from every tissue, both *cpg2* and *cpg2b* were amplified only from brain cDNA (Figure 2-3A), indicating that *cpg2* and *cpg2b* are brain-specific splice-variants of *syne-1*. *In situ* hybridization of an adult rat brain using a probe primarily against the *cpg2* 3'UTR showed that *cpg2* is expressed in the cerebral cortex, hippocampus, striatum, and cerebellum of adult rats (Figure 2-3B).

Thus, the expression of *cpg2* and *cpg2b* is localized specifically to brain regions with robust activity-dependent synaptic plasticity mechanisms.

CPG2 protein analysis

Consistent with its sequence derived from the separator region of the *syne-1* gene, the CPG2 and CPG2B protein sequences contain several motifs that indicate a structural or protein-interaction function. Figure 2-4A shows the rat CPG2 and CPG2B amino acid sequence, highlighting the location of two spectrin repeats and several coiled-coil motifs, primarily clustered towards the C-terminus. Both of these domains can underlie protein dimerization and protein-protein interactions (Burkhard et al., 2001; Djinovic-Carugo et al., 2002). Thus, CPG2 contains sequences that suggest it may organize a protein network. Also marked are the monoclonal anti-CPG2 antibody antigen and the location of a downstream methionine that may be the site of leaky transcription initiation of *cpg2*, as discussed below.

To study the CPG2 protein, an anti-CPG2 monoclonal antibody (200A6.1) was generated against a peptide from CPG2 (Figure 2-4B). When used to probe a Western blot of rat cerebral cortex extracts, this antibody recognized a doublet band at approximately 100 and 110 kD, close to the predicted size of the protein from both *cpg2* transcripts (Figure 2-5A, lane 1). Protein extracts from 293T cells transfected with *cpg2* cDNA probed with the same antibody showed the same sized doublet band as brain extracts (Figure 2-5A, lane 2), suggesting that both bands of the *in vivo* CPG2 doublet are generated from the translation of the *cpg2* transcript. The band from 293T cell extracts was competed away by preadsorbing the antibody with the immune peptide (Figure 2-5A, lane 3), showing the specificity of the antibody.

To define the source of the CPG2 doublet, an HA-*cpg2* construct, in which an HA tag was fused to the N-terminus of CPG2, was generated and transfected into 293T cells. Protein extracts from these cells were then immunoprecipitated with either an anti-HA antibody or the anti-CPG2 monoclonal antibody 200A5, separated on an SDS-PAGE gel, and visualized with a silver stain (Figure 2-5B). The anti-CPG2 antibody immunoprecipitated a doublet band, while the anti-HA antibody immunoprecipitated a single band the same size as the upper band immunoprecipitated with the anti-CPG2 antibody. The upper band appears to be the full-length fusion protein, while the lower band is likely a truncated version of CPG2 which does not contain the N-terminal HA tag (Figure 2-5B, top). This truncated CPG2 may result from cleavage of the full-length protein or the leaky translation initiation of the *cpg2* transcript, indicating that a similar process underlies the formation of the *in vivo* CPG2 doublet band. Interestingly, there is an ATG translation initiation codon 70 codons downstream of the CPG2 start site (Figure 2-4). A protein translated from this site would be 101 kD, the approximate size of the lower band of the doublet band. In addition, it is possible that the translation of *cpg2b* is also leaky and its protein begins with the downstream ATG sites that would result in the equivalent proteins as CPG2.

Activity-regulation of *cpg2* transcription and translation

Previous studies showed that *cpg2* expression is regulated by activity (Nedivi et al., 1993; Nedivi et al., 1996). However, these studies did not indicate if there was a corresponding increase in CPG2 protein following *cpg2* induction. To confrim the induction of *cpg2* by activity, adult rats were given seizures by intraperitoneal injections of kainic acid. After 6 hours, the cortices and hippocampi were harvested and their RNA extracted. Northern

blots of total RNA from these and control tissues were performed. They showed that there was a >4-fold increase in *cpg2* transcript levels in the hippocampus and >2.5-fold increase in the cortex following seizure induction (Figure 2-6A). To determine if there were similar changes in CPG2 protein levels following seizure induction, protein from the hippocampi and cortices of kainic-acid injected rats was collected 24 hours after seizure induction and probed by Western blot. There were no changes in CPG2 protein levels in either brain area at this time point following seizure (Figure 2-6B). Protein was extracted from hippocampi of seizure-induced rats collected at increasing time points following injection to confirm there were no transient CPG2 protein level changes. At each time point, there were no changes in CPG2 protein levels (Figure 2-6C). Thus, while there is a multiple-fold increase in *cpg2* transcript levels, there is no corresponding increase in CPG2 protein levels following seizure. In the Discussion, I address several potential mechanisms that may underlie this discrepancy.

CPG2 localizes to postsynaptic sites of excitatory synapses

A primary means towards illuminating the function of a protein is to determine its subcellular localization. To this end the anti-CPG2 monoclonal antibody 200A6.1 was used to stain cultured hipppocampal neurons. The resulting staining pattern showed a punctate distribution of CPG2 along the length of dendrites (Figure 2-7A). To confirm the specificity of the antibody for the CPG2 protein, a lentivirus that expresses a CPG2-GFP fusion protein was used to infect cultured hippocampal neurons, which were then stained for CPG2 (Figure 2-7B). The GFP and antibody staining patterns overlapped, showing that the anti-CPG2 antibody specifically recognizes the CPG2 protein. The fact that *cpg2* is a splice-variant of a much larger gene raises the possibility that the anti-

CPG2 antibody may recognize another *syne-1* derived protein. However, Northern and Western blots show no other bands in the brain, and an antibody against the C-terminus of Syne-1 (Apel et al., 2000) showed no staining of hippocampal neurons (data not shown). Thus, the protein recognized by the anti-CPG2 antibody in hippocampal neurons corresponds to the protein encoded by the *cpg2* transcript.

The punctate distribution of CPG2 along neurites is indicative of a synaptic localization for CPG2. To determine if CPG2 is indeed localized to synapses, cultured neurons were filled with GFP via lentivirus infection and labeled for CPG2 and synapsin, a marker for presynaptic terminals. CPG2 was found to localize to most but not all synapsin-positive synapses, often on dendritic spines (Figure 2-7C). Thus, CPG2 is localized specifically to a subset of synaptic sites in hippocampal neurons. There are two types of synapses in hippocampal cultures: GABAergic/inhibitory synapses and glutamatergic/excitatory synapses. To identify the subtype of synapse to which CPG2 is localized, GFP-filled hippocampal neurons were stained for CPG2 and GAD65, a marker for inhibitory presynaptic terminals. There was little correspondence between GAD65 and CPG2 staining (Figure 2-8A), suggesting that CPG2 is not localized to inhibitory synapses. To confirm its localization to excitatory synapses, GFP-filled cells were stained for CPG2 and PSD-95, a postsynaptic marker of excitatory synapses. CPG2 staining was apparent on PSD-95-positive synapses on dendritic spines and shafts (Figure 2-8B). Thus, CPG2 is specifically localized to excitatory synapses on both dendritic shafts and spines of hippocampal neurons.

Close examination of the CPG2 and PSD-95 staining patterns revealed that CPG2 does not colocalize with PSD-95 within dendritic spines, indicating that CPG2 is not part

of the postsynaptic density. To define further the localization of CPG2 in dendritic spines, cultured hippocampal neurons were stained for CPG2, PSD-95 and actin filaments, the major cytoskeletal component of dendritic spines. In this triple stain, CPG2 colocalized with neither actin filaments nor PSD-95 (Figure 2-9A), indicating that CPG2 does not localize to either the actin-rich region or the postsynaptic density of dendritic spines. To confirm its localization to a subdomain of dendritic spines, pre-embedding anti-CPG2 immunoEM was performed on rat brain sections and cultured hippocampal neurons. Electron micrographs of rat dentate gyrus dendritic spines showed that CPG2 is localized lateral to and beneath the postsynaptic density in both spine and shaft synapses *in vivo* (Figure 2-9B) and *in vitro* (Figure 2-9C). Postembedding immnogold EM confirmed CPG2's localization relative to the PSD and, additionally, showed that it is found underneath NMDA receptors from adjacent serial sections (Figure 2-9D). Thus, CPG2 is indeed localized to a unique subdomain of excitatory synapses and dendritic spines, segregated from the postsynaptic density and the actin-rich core of spines.

Recent evidence has shown that excitatory synapses contain a postsynaptic endocytic zone, distinct from the postsynaptic density, that is labeled by a GFP-clathrin A1 light chain fusion protein (Blanpied et al., 2002). Its distribution within dendritic spines is similar to that of CPG2. Therefore, this clathrin-GFP construct was subcloned into a lentivirus transfer vector, and hippocampal neurons were infected with the resulting virus and stained for CPG2 and PSD-95. When viewed at low magnification, clathrin-GFP can be seen in the dendritic shaft and in a punctate pattern in the dendritic spines that are also positive for CPG2 and PSD-95 (Figure 2-10A). High magnification images of individual spines from these neurons showed that CPG2 and clathrin-GFP

staining overlap significantly with each other, but not with PSD-95 staining. These data suggest that CPG2 is localized to the endocytic zone of dendritic spines.

Since CPG2 is localized to the endocytic zone of dendritic spines and synapses, CPG2 may associate with clathrin-coated pits and/or vesicles within that zone. Examination of EM micrographs of CPG2 staining from cultured hippocampal neurons showed that CPG2 does indeed localize to synaptic regions containing endocytic pits (Figure 2-10C). However, synapse-associated clathrin-coated pits were rare. When cultured neurons were treated with the actin depolymerizing agent latrunculin A, there was a dramatic increase in the number of endocytic pits at synaptic sites. CPG2 staining was often associated with those pits (Figure 2-10D). Thus, CPG2 is localized to the postsynaptic endocytic zone in close proximity to clathrin-coated pits, raising the possibility that CPG2 functions in the regulation of synaptic protein internalization.

Discussion

In this Chapter, I show that the activity-regulated transcript *cpg2* is one of two closely-related splice-variants of the *syne-1* gene whose expression is restricted to brain regions capable of synaptic plasticity. Although the *cpg2* transcript is upregulated multiple-fold in the hippocampus and cortex following seizure induction, the level of the CPG2 protein remains relatively constant. Furthermore, I show that CPG2 specifically localizes to the postsynaptic site of excitatory synapses, where it associates with clathrin-coated pits at the postsynaptic endocytic zone. These data suggest that CPG2 is a regulator of clathrin-mediated endocytosis of postsynaptic proteins, unique to excitatory synapses in regions capable of robust synaptic plasticity.

cpg2* is a splice-variant of *syne-1

The *cpg2* transcript is a 5.9 kB splice-variant of the *syne-1* gene. *syne-1* encodes a large protein (>1000 kD) that contains a nuclear localization domain at its C-terminus and an actin-binding domain at its N-terminus, which are separated by a long separator region that contains multiple spectrin repeats, two Golgi binding domains, and a kinesin binding domain (Fan and Beck, 2004; Gough et al., 2003; Starr and Han, 2003). First isolated in a screen for acetylcholine receptor interacting proteins in muscles (Apel et al., 2000), Syne-1 is thought to anchor the nucleus to the actin cytoskeleton, and possibly form a complex between the cytoskeleton, nucleus, and Golgi complex (Gough et al., 2003; Starr and Han, 2003). It has recently been found that Syne-1 facilitates the trafficking of vesicles at the spindle midbody during cytokinesis (Fan and Beck, 2004). These data suggest that Syne-1 may regulate membrane trafficking, raising the possibility that CPG2 performs a similar function in neurons. Several splice-variants of *syne-1* containing either the N- or C-terminus have been reported, all of which are either not expressed or have a low expression level in the brain (Apel et al., 2000; Zhang et al., 2001).

There are two potential versions of the *cpg2* transcript (*cpg2* and *cpg2b*). They correspond to 19 (*cpg2*) or 20 (*cpg2b*) exons from the alpha-helical separator region of *syne-1* and do not contain exons encoding the actin-binding domain, the nuclear localization domain, or the Golgi binding motifs. How do the *cpg2* splice-variants arise? The genomic DNA sequence upstream of the transcription start sites suggests that there are several potential regulators of *cpg2* and *cpg2b* transcription. However, what regulates their initiation is still unclear. Meanwhile, the mechanisms of early transcription and translation stop are evident from the *cpg2* sequence. The intron between exons 34 (the

final coding exon of *cpg2*) and 35 of *syne-1*, is not spliced, leaving 7 additional codons, including the translation stop codon. Following exon 35 of *syne-1*, there is a non-canonical polyadenylation hexamer (ATTAAA), which is likely the site of pre-mRNA cleavage and poly-A addition. Interestingly, this polyadenylation hexamer is commonly found in early truncation splice-variants of genes (MacDonald and Redondo, 2002), including *homer*, where it regulates the transcription termination of the *homer 1a* activity-regulated, truncated transcript of the *homer* gene (Bottai et al., 2002). It is interesting to speculate that the signaling pathways that regulate the early transcription termination of the *homer1a* and *cpg2* transcripts may be a locus of activity-regulation.

Consistent with its derivation from the *syne-1* separator region, the CPG2 protein sequence contains domains common to structural proteins, including two spectrin repeats and several coiled-coil motifs. Both of these domains are known to be involved in protein homo- and heterodimerization, through which they can mediate the organization of protein complexes (Burkhard et al., 2001; Djinovic-Carugo et al., 2002). Historically thought to regulate cellular structure, proteins of the spectrin family have recently been shown to function as accumulators of proteins that may serve to anchor networks of cytoskeletal, signal transduction, and transmembrane proteins (Pinder and Baines, 2000). The presence of these structural motifs suggests that the CPG2 protein may regulate the localization of proteins via its spectrin repeats and/or coiled-coils domains.

Activity-regulation of *cpg2* transcription and translation

From Northern blot analysis, the *cpg2* transcript was found to increase by greater than 4-fold in the rat hippocampus following a kainic-acid induced seizure, confirming its previous characterization as an activity-regulated transcript. However, there was no

corresponding increase in CPG2 protein levels following seizure. The mechanism underlying this dichotomy is unclear. One possibility is that, during periods of activity, the stability of CPG2 is decreased, resulting in greater turnover of the protein. The reason *cpg2* expression increases is to balance this use-dependent turnover of CPG2 during plasticity. A second possibility is that the extra *cpg2* copies are translationally silent and require a signal to unmask their translation that may not be present or may be saturated. A related possibility is that *cpg2* is dendritically translated. The 3'UTR contains a cytoplasmic polyadenylation element (CPE) immediately downstream of the polyadenylation hexamer (see Figure 2-1C). CPEs are bound by CPE binding protein (CPEB), which induces polyadenylation of the transcript and, as a result, its translation (Mendez and Richter, 2001). CPEB is required for dendritic localization of several transcripts, suggesting that it also functions as an mRNA trafficking protein (Huang et al., 2003). It is possible that the *cpg2* transcript is upregulated by activity and trafficked to synaptic sites, where local cues can initiate local translation of the *cpg2* transcript. Such a mechanism would likely generate only small amounts of additional protein, resulting in no detectable increases in protein levels following seizures by Western blot.

CPG2 localizes to the postsynaptic endocytic zone

Glutamate receptors undergo constitutive internalization (Ehlers, 2000; Lin et al., 2000), and long-term depression (LTD) likely results, in part, from the internalization of synaptic glutamate receptors (Carroll et al., 2001). Glutamate receptor internalization is thought to occur through clathrin-mediated endocytosis (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Man et al., 2000; Snyder et al., 2001), a general mechanism for the internalization of proteins from the plasma membrane (Mousavi et al., 2004). An

endocytic zone has recently been identified at the postsynaptic side of excitatory synapses (Blanpied et al., 2002). Marked by a clathrin-GFP fusion protein, this endocytic zone, segregated from the PSD, is stable and formed independently of activity, suggesting it is a fundamental spine component. Although it has not been shown directly, glutamate receptor internalization likely occurs at the postsynaptic endocytic zone (Blanpied et al., 2002). CPG2 localizes specifically to an endocytic zone of excitatory synapses, often in the vicinity of clathrin-coated pits, raising the possibility that CPG2 may play a role in the regulated endocytosis of synaptic proteins, including glutamate receptors. Its protein interaction motifs suggest that it may function by organizing a protein complex at the endocytic zone.

Methods

Northern blot

Northern blot hybridization was done as described (Sambrook et al., 1989). Total RNA from rat cerebral cortex (25 μg) was separated on a 1% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with ^{32}P-labeled probes using stringent conditions. Probes were synthesized with the High Prime labeling kit (Roche) using a 1 kb fragment from the coding region of rat *cpg2* or a 300 bp mouse *GAPDH* cDNA fragment excised from pTRI-GAPDH-mouse (Ambion). Rat seizures were induced by intraperitoneal injections of kainic acid (10 mg/kg; Sigma) and tissue was extracted 6 hours later for RNA extraction and 0 to 24 hours later for protein extracts as described below.

5' RACE

cpg2-5' ends were generated by 5' RACE with the GeneRacer Kit (Invitrogen), using a *cpg2* gene specific primer (GCTCCACGGACTCTCGCCGCCCATA). These 5' fragments were amplified by PCR using a sense primer specific to the ligated linker and a nested primer specific to *cpg2* (GACTCAGCGAGGACCAGCAGGGACAGTA). In the negative control, RNA was processed identically, except that the reverse transcriptase was omitted during the original 5' RACE reaction. The two PCR products were subcloned using the TOPO TA Cloning Kit (Invitrogen) and sequenced (Retrogen).

RT-PCR

Tissue from Sprague-Dawley rats was frozen on dry ice and ground into a fine powder with a mortar and pestle. Total RNA was extracted using Trizol (Invitrogen). cDNA was synthesized using total RNA with cDNA Sythesis Kit (Invitrogen). Fragments of cDNA transcripts were amplified via PCR (30 cycles) using 0.5 µl of the cDNA reaction and *taq* DNA polymerase (Roche). For *cpg2* and *cpg2b* the same antisense primer was used (CCATCGGCTTTGACGTACAT) with unique sense primers (*cpg2*: AAAGGCTTCCATCGGTCGTT; *cpg2b*: TCAGCCTCTTGTTTCCTCGT). Control reactions used primers against *β-actin* (sense: AAATGCTGCACTGTGCGGC; antisense: GTTTTATAGGACGCCACAGC). Sense and antisense primers were always from separate exons to avoid amplification of genomic DNA.

***In situ* hybridization**

In situ hybridization was as described (Lee and Nedivi, 2002) on saggittal sections of adult rat brain using a probe generated from the 3' UTR of the *cpg2* transcript.

Protein extracts, antibody generation, and Western blot

Cerebral cortex protein extracts were generated by homogenizing rat hippocampi in 10 mM Tris (pH 7.4), 1 mM EGTA, 0.5 mM DTT plus a protease inhibitor cocktail (1:200; Sigma). Protein concentrations were calculated using Bradford (Pierce). HEK293T-*cpg2* protein extracts were generated from HEK293T cells transfected with *cpg2*-pcDNA3 (Promega) using Lipofectamine2000 (Invitrogen). After 24 hours, cells were rinsed and lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, and 0.5% NP-40 (Sigma). Cell lysates were centrifuged at 14000 RPM for 15 min and supernatants were diluted to 50% in 2X SDS-PAGE sample buffer.

Mouse monoclonal anti-CPG2 antibodies 200A5 (IgG_{2a}) and 200A6.1 (IgG_1) were generated against the CPG2-specific peptide RKLESTLTGLEQSRERQERR (ResGen). Antibodies were purified from hybridoma supernatants with Protein G columns (Invitrogen). Protein extracts from rat cerebral cortex (40 μg) and HEK293T cells were separated on an 8% SDS-PAGE gel, transferred to nitrocellulose, and blocked with 10% non-fat milk. One lane of hippocampal extracts and one lane of HEK293T cell extracts were probed for 1 hour at room temperature with the monoclonal anti-CPG2 antibody 200A6.1 (3 μg/ml) and an anti-actin monoclonal antibody (1:500; Sigma), while one lane of HEK293T cell extracts was probed with the monoclonal antibody preadsorbed with the immune peptide (50 μg/ml). The blot was washed in TBS, incubated in horse radish peroxidase-conjugated goat anti-rabbit secondary antibodies (Sigma; 1:5000), rinsed in TBS, and visualized with ECL (Amersham).

293T cell immunopreciptiation and silver stain

An *HA-cpg2* fusion sequence was subcloned into the pcDNA3 (Promega) mammalian expression vector downstream of the CMV promoter. 293T cells in 15 cm dishes were tranfected with *HA-cpg2* or *pEGFP-C1* with Lipofectamine2000. After 24 hours, cells were lysed in ice-cold NP-40 buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.5% NP-40), collected into Eppendorf tubes, and incubated on ice for 20 min. Tubes were centrifuged at 14000 RPM for 15 min at 4°C, and supernatants were collected. IP antibody was added (5 μg) and tubes were incubated for 1 hr at 4°C with rocking. Antibodies used were anti-CPG2 monoclonal 200A5 or rabbit polyclonal anti-HA (Santa Cruz). Protein A-agarose slurry (20 μl; Sigma) was added and tubes were incubated overnight at 4°C with rocking. Agarose beads were washed with cold NP-40 buffer 3 times. After the last wash, beads were resuspended in 15 μl 1X SDS-PAGE loading buffer and boiled for 5 min. Samples were run on an 8% SDS-PAGE gel. The gel was then fixed and silver stained with GelCode SilverSnap Stain Kit (Pierce).

Hippocampal cultures

Hippocampal cultures were made essentially as described (Brewer et al., 1993). Glass coverslips (Carolina Biological Supply) in culture dishes were incubated in poly-D-lysine (40 μg/ml) and laminin (2.5 μg/ml; BD Biosciences) overnight, rinsed 3 times with water, and incubated in plating medium: Neurobasal (Gibco) supplemented with 0.5 mM L-glutamine, 12.5 μM L-glutamate, and B27 (Gibco). Hippocampi from E18 embryos were isolated, digested in 0.25% trypsin (Gibco) plus 0.1% DNAse (Sigma), and triturated with fire-polished pipettes in 0.1% DNAse. Cells were then centrifuged at 1000 RPM for 10 min at 4°C, resuspended in plating medium, counted, and plated at 1.6 X 10^4

cells/cm^2. After 4 days, one half of the medium was replaced with plating medium without additional L-glutamate.

Immunocytochemistry and fluorescent microscopy

Cultures were fixed in 4% formaldehyde at 4°C for 20 min, permeabilized in 0.3% Triton-X-100/PBS for 5 min at 4°C, and blocked in 10% goat serum in PBS for 1 hour at 4°C. Cultures were then incubated overnight in primary antibodies in PBS at 4°C, rinsed 3 times in PBS, and incubated in secondary antibodies for 1 hour at room temperature. Following 3 washes in PBS, cultures were mounted onto slides with Flouromout G (Southern Biotechnology). Primary antibodies were as follows: mouse anti-CPG2 monoclonal 200A6.1 (1 μg/ml), rabbit anti-synapsin (1:2000; Sigma), rabbit anti-PSD-95 (1:300; Zymed), rabbit anti-GAD65 (1:2000; Chemicon), and rabbit anti-Syne-1 (Apel et al., 2000). Secondary antibodies were as follows: goat anti-mouse Alexa Flour555 (1:1000; Molecular Probes) and goat anti-rabbit Alexa Flour647 (1:500; Molecular Probes). For actin filament staining, Alexa Flour488-conjugated phalloidin (1:500; Molecular probes) was added to the secondary antibody mixture. Flourescent images of neurons were imported into Spot 3.5 (Diagnostic Instruments) with a Diagnostic Instruments Spot2 digital camera mounted on a Nikon Eclipse E600 using a 60X/1.40 Plan Apo oil immersion objective (Nikon). Images were saved as gray-scale TIFFs and overlayed and pseudo-colored for figures with Photoshop5.0.

Lentivirus generation

The lentivirus transfer vector pFUGW, in which GFP expression is driven by the human ubiquitin-C promoter, was used to generate virus particles for filling neurons with GFP

(Lois et al., 2002). For *cpg2-gfp* lentivirus generation, the *cpg2* sequence was subcloned downstream of EGFP in EGFP-C1 (Clontech). The *cpg2-gfp* sequence was then subcloned downstream of the ubiquitin-C promoter in pFUGW. The clathrin light chain A1-GFP fusion sequence from EGFP-C1 (Blanpied et al., 2002) was subcloned into the pFUGW lentivirus vector downstream of the ubiquitin-C promoter. Lentivirus particles containing these vectors were produced by cotransfecting the transfer vector, the HIV-1 packaging vector Δ8.9, and the VSVG envelope glycoprotein into 293T cells with Lipofectamine2000 (Invitrogen) 24 hours after plating at 15 X 10^6 cells/15 cm culture dish. 48-60 hours after transfection culture media was collected and centrifuged at 5000 RPM for 5 min. Media was filtered and centrifuged at 25000 RPM for 90 min at 4°C. Supernatants were discarded, and the virus pellets were rehydrated in PBS at 4°C overnight. Viral particles were then resuspended, aliquoted, and frozen at -80°C. Viral titers were determined by application of serial dilutions to 293T cells and quantifications of GFP expression 72 hours later. Typical titers were ~1X10^6 pfu/μl.

Immunoelectron microscopy

Hippocampal cultures or vibratome sections of perfusion-fixed rat hippocampi were processed for CPG2 or NR1 immunostaining for electron microscopic analysis. Cultures were immersion fixed with 4% paraformaldehyde and 0.1% glutaraldehyde. Cultures were then immunostained for CPG2 (1 μg/ml) or NR1 (1:1000; Chemicon) overnight at room temperature. After several washes in PB, section were incubated with biotinylated horse anti-mouse secondary antibodies (1:250; Vector) for 2 hours followed by incubation in Avidin biotin-peroxidase (1:200; Vector) for 2 hours at room temperature. Tissue-bound peroxidase was then visualized with a diaminobenzidine (DAB; 5 mg/ml,

H_2O_2 0.03%) reaction. Coverslips were osmicated, dehydrated and embedded in durcupan (FLUKA, ACM). Ultrathin sections (60 nm thick) were prepared on a Ultracut UCT, Leica ultramicrotome and analyzed in a Tecnai 10 electron microscope.

For CPG2 and NR1 postembedding immunoEM, lowicryl-embedded hippocampal sections were used as previously described (Nyiri et al., 2003). Sections were washed in PB overnight and then placed into increasing concentration sucrose solutions (0.5-, 1-, and 2-M sucrose for 0.5, 1 and 2 hrs, respectively) for cryoprotection. Sections were then slammed onto copper blocks cooled in liquid nitrogen and, following low-temperature dehydration and freeze-substitution, embedded in Lowicryl HM 20 resin (Chemische Werke Lowi). Postembedding immunocytochemistry was carried out on 60-nm-thick serial sections placed on pioloform-coated nickel grids. Serial sections on two consecutive grids were incubated on drops of blocking solution for 1 h (20% bovine serum albumin), followed by incubation on drops of either anti-CPG2 or anti-NR1 primary antibodies overnight at room temperature. The primary and secondary antibodies (15 nm gold-conjugated anti-mouse IgG, 1:20; Amersham) were diluted in TBS plus 0.03% Triton X-100 and 2% human serum albumin (Sigma). Following several washes in TBS, the sections were washed in ultrapure water, and then contrasted with saturated aqueous uranyl acetate followed by lead citrate.

Figure 2-1. *cpg2* is a splice-variant of *syne-1*

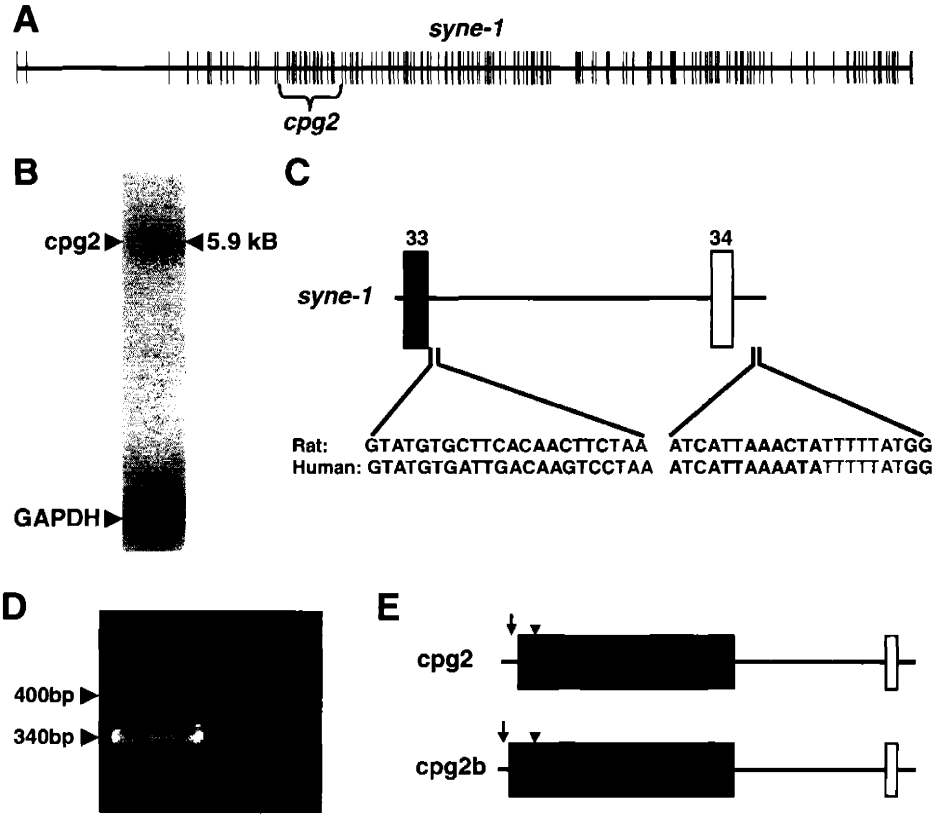

Figure 2-2. *cpg2* and *cpg2b* transcription initiation

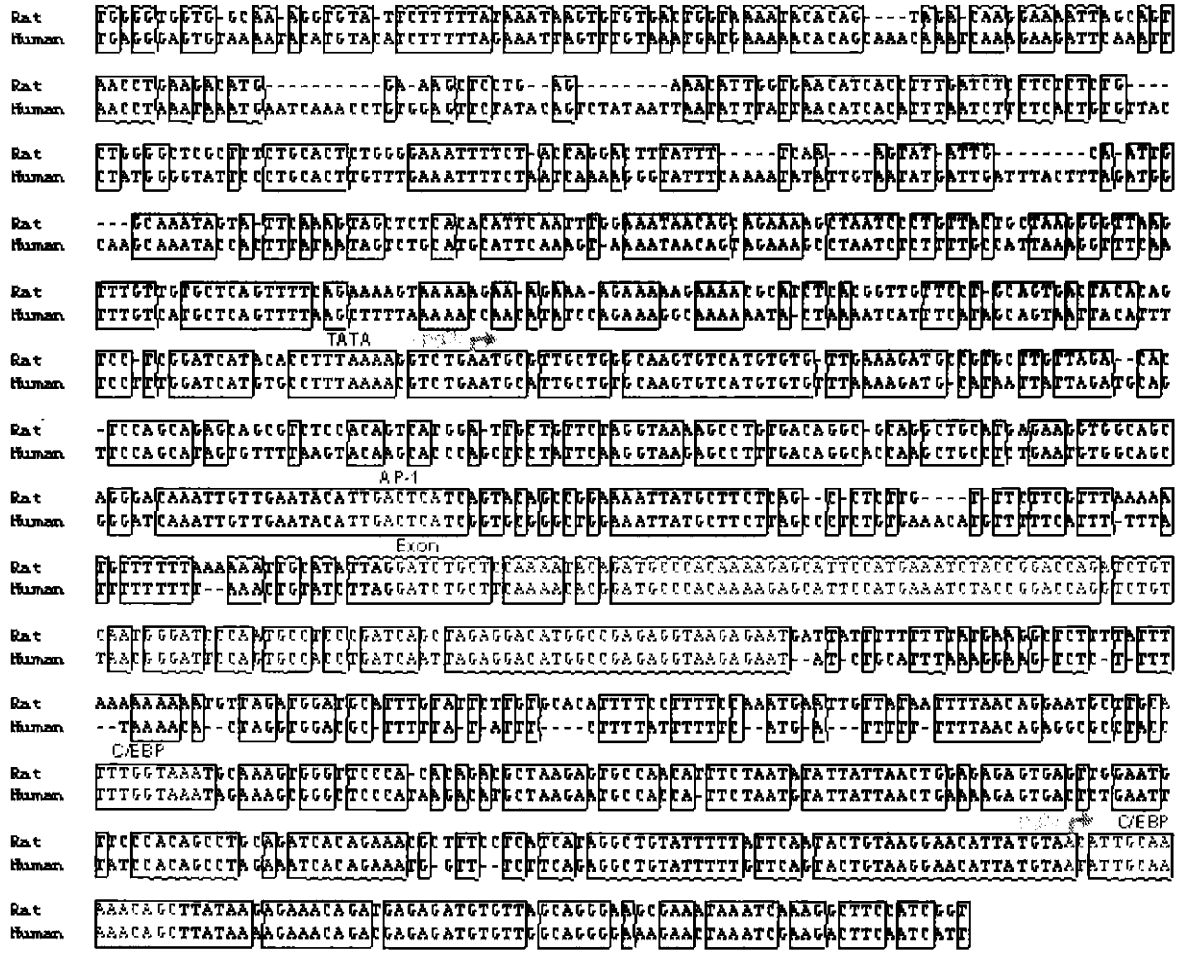

Figure 2-3. *cpg2* expression is limited to brain regions capable of synaptic plasticity

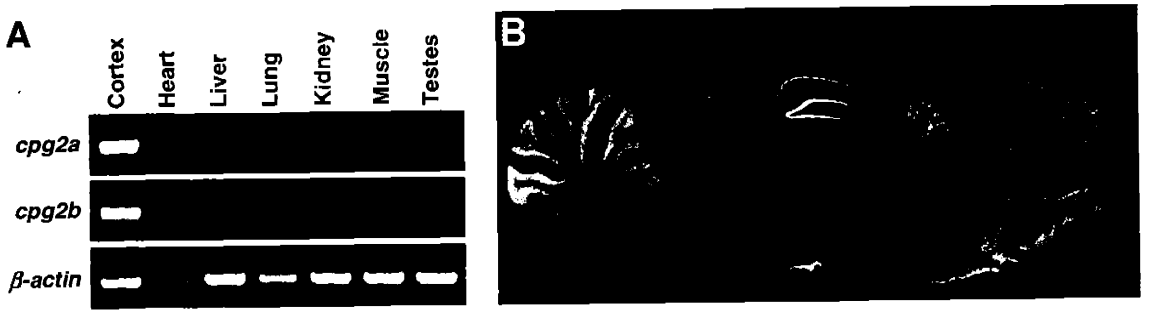

Figure 2-4. CPG2 protein structure

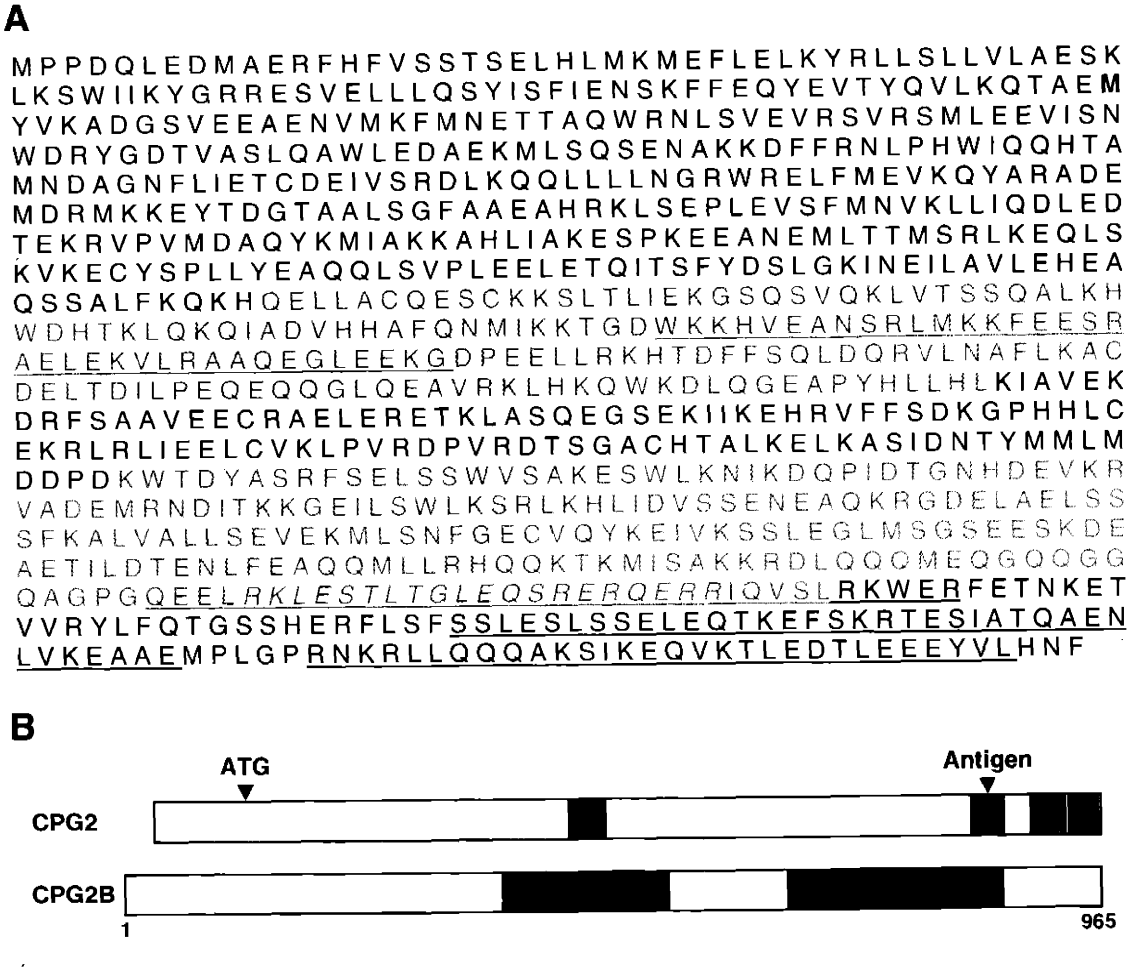

Figure 2-5. CPG2 protein analysis

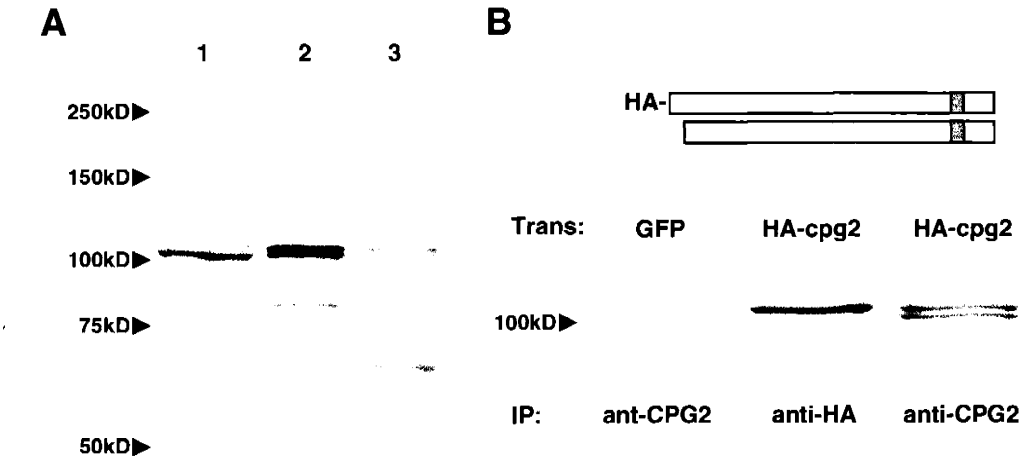

Figure 2-6. Activity-regulation of *cpg2* transcript and CPG2 protein levels

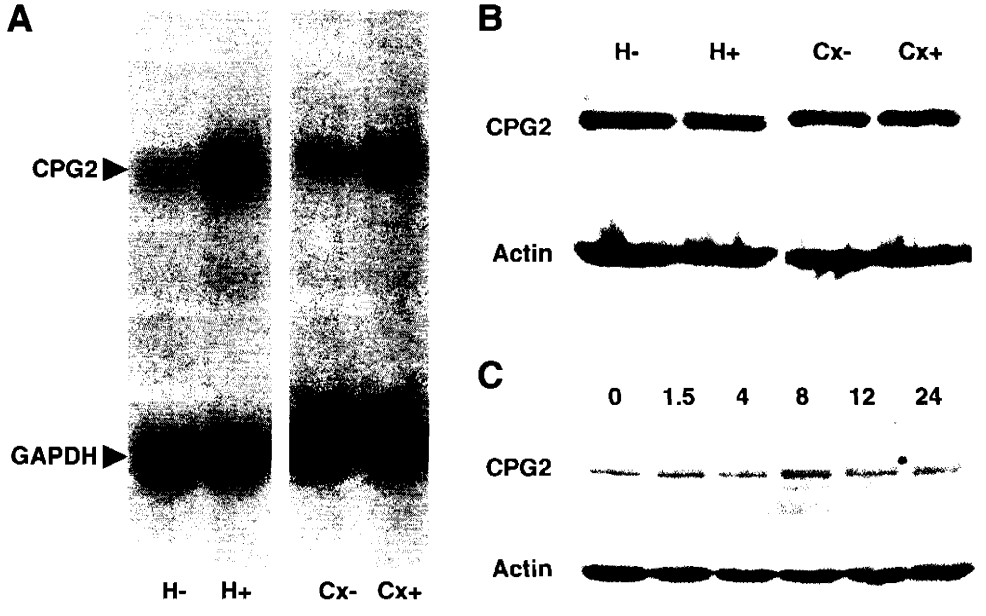

Figure 2-7. CPG2 localizes to synapses in cultured hippocampal neurons

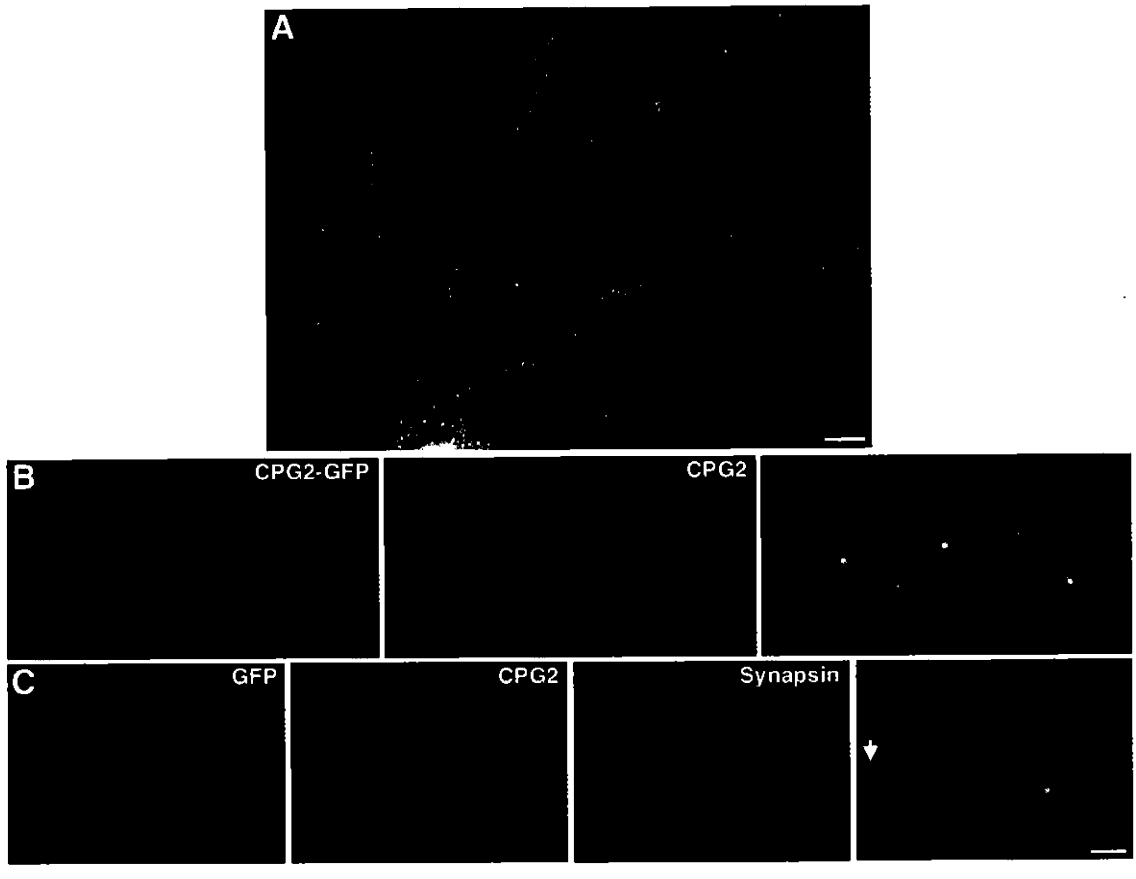

Figure 2-8. CPG2 localizes to excitatory synapses

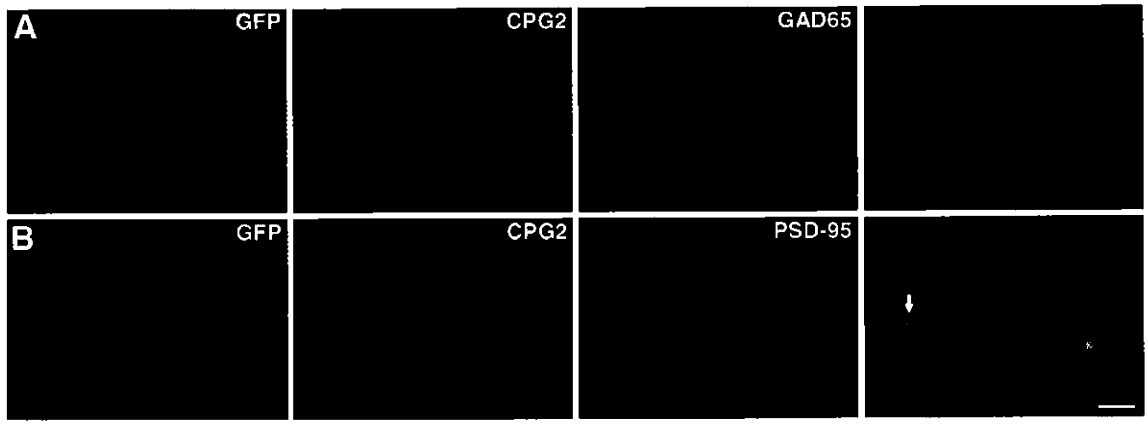

Figure 2-9. CPG2 localizes to a subdomain of excitatory synapses

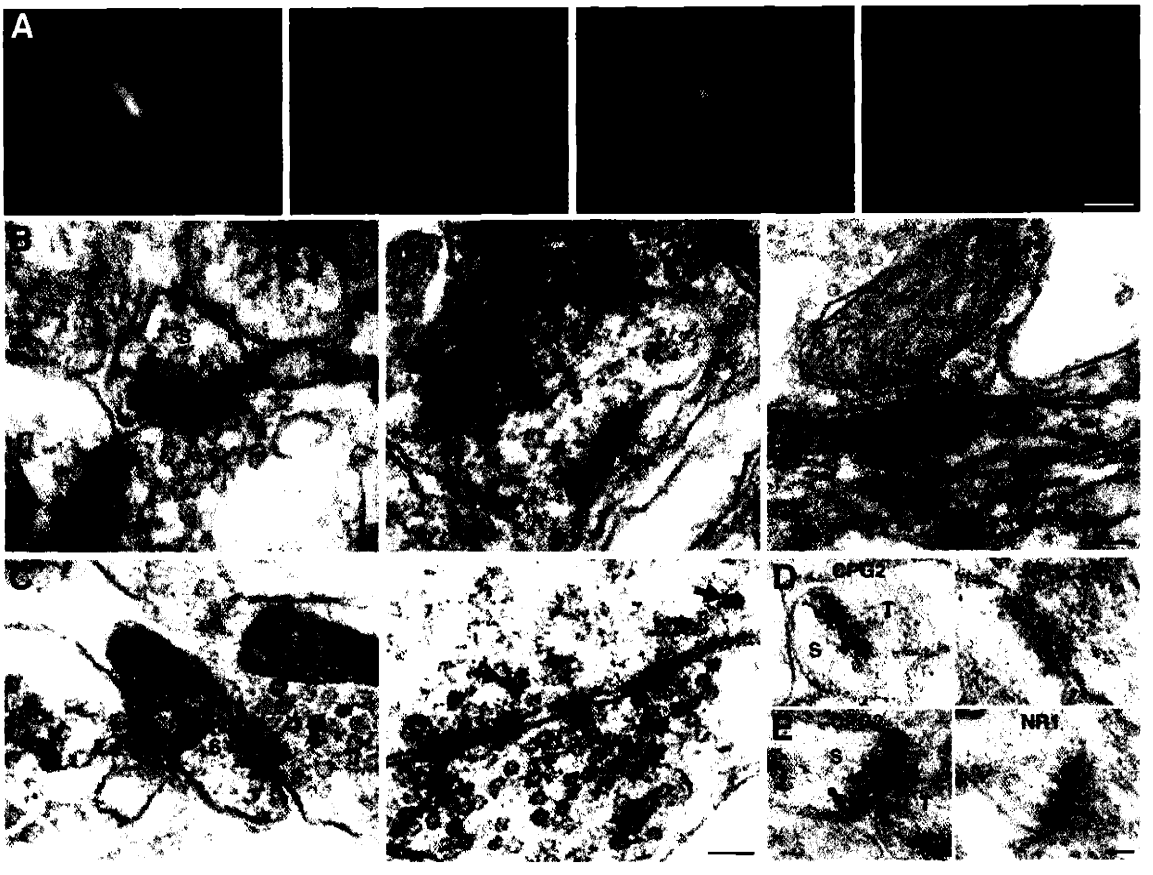

Figure 2-10. CPG2 localizes to an endocytic zone of excitatory synapses

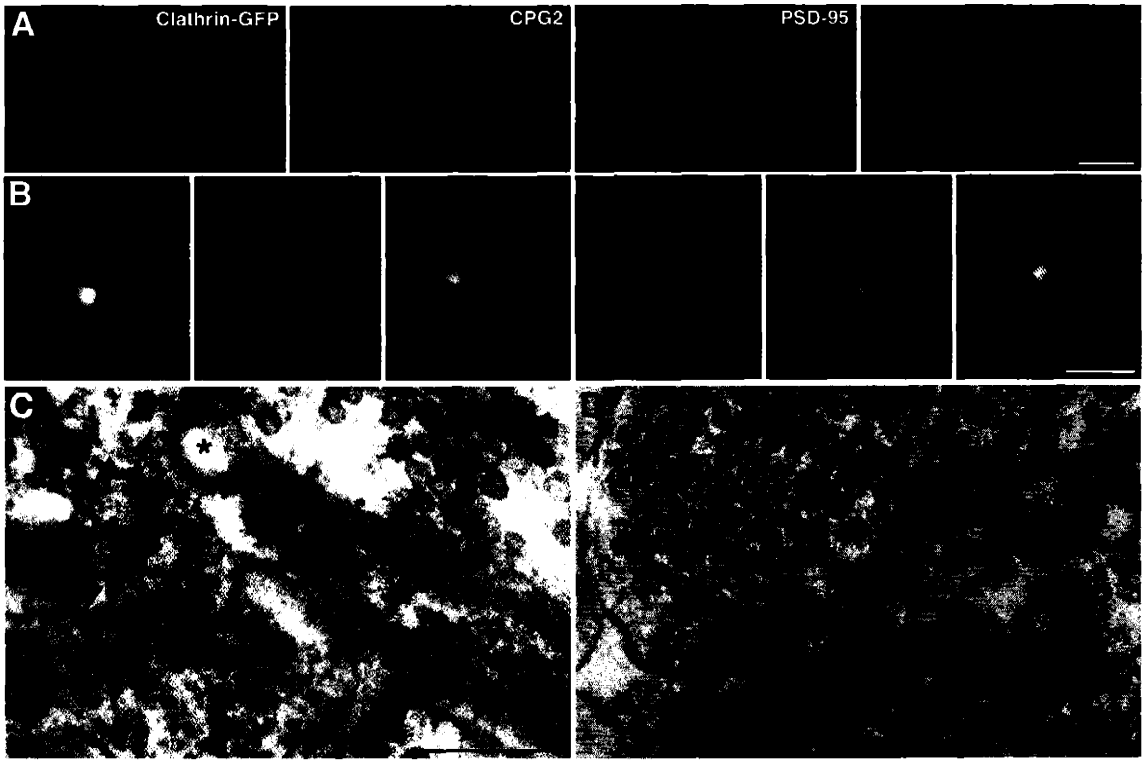

Figure 2-1. *cpg2* is a splice-variant of *syne-1*

(A) Schematic of the *syne-1* gene with exons marked by vertical lines and introns by horizontal lines. Red lines indicate exons corresponding to the coding region of the original *cpg2* transcript. (B) Northern blot of rat brain total RNA probed with a 1 kB fragment of the *cpg2* coding region reveals a 5.9 kB transcript. (C) Schematic of the *syne*-1 genomic DNA sequence at the site of *cpg2* transcription and translation termination. The final exon in the *cpg2* coding region is marked by a red box, and a non-coding exon contained in the 3' UTR is marked in yellow. *syne-1* exon number is shown above the boxes. Conserved rat and human sequences following the final coding exon shows the translation stop codon (green). Sequence following the non-coding exon shows the polyadenylation hexamer (red), the site of *cpg2* transcription termination, and a potential CPE (blue). (D) 5' RACE products of rat brain total RNA using a primer in the first *cpg2* coding exon with (left) or without (right) reverse transcriptase, followed by PCR using nested primers. (E) Schematic of two potential *cpg2* splice-variants identified by sequencing the 5'RACE fragments. The green box indicates an additional exon spliced to the 5' end of the *cpg2* transcript. Arrows indicate the locations of the RT-PCR primers (see Figure 2-3A).

Figure 2-2. *cpg2* and *cpg2b* transcription initiation

Rat and human genomic DNA sequences showing the sites of *cpg2* and *cpg2b* transcription initiation (orange). Also highlighted are a potential TATA box (red), potential transcription factor binding sites (green), and the first exon in *cpg2b* (blue).

Figure 2-3. *cpg2* expression is limited to brain regions capable of synaptic plasticity

(A) Tissue RT-PCR from total RNA using primers specific to *cpg2*, *cpg2b*, and *β-actin*. (B) *In situ* hybridization on a saggital section through an adult rat brain using a probe unique to *cpg2* and *cpg2b*.

Fig. 2-4. CPG2 protein structure

(A) Rat CPG2 protein sequence showing additional residues in CPG2B (blue), potential downstream translation start methionine (red), spectrin repeats (green), coiled-coil regions (underline), and the antibody antigen (italics). (B) Schematic diagrams of CPG2 and CPG2B showing location of the first downstream ATG, the four predicted coiled-coils (blue boxes), the two spectrin repeats (red boxes), and the location of the antibody antigen (orange bar).

Figure 2-5. CPG2 protein analysis

(A) Western blot of protein extracts from rat cerebral cortex (lane 1) or 293T cells expressing *cpg2* (lanes 2+3) probed with the anti-CPG2 monoclonal antibody 200A6.1 (lanes 1+2). Lane 3 was probed with the monoclonal antibody preabsorbed with the immune peptide (50 μg/ml). (B) Silver stain of an SDS-PAGE gel of protein extracts from 293T cells transfected with the indicated plasmids and immunoprecipitated with the indicated antibodies. Top: The predicted structure of the HA-tagged CPG2 protein in 293T cells showing the site of anti-CPG2 (blue box) and anti-HA antibody antigens.

Figure 2-6. Activity-regulation of *cpg2* transcript and CPG2 protein levels

(A) Northern blot of total RNA from the hippocampus (H) or cerebral cortex (Cx) of control rats (-) or rats six hours following seizure induction by kainic acid injection (+) probed for *cpg2* and *GAPDH*. (B) Western blots of protein extracts from the hippocampus or cortex of control rats or rats 24 hours following seizure induction by kainic acid injection probed with antibodies against CPG2 and actin. (C) Western blots of protein extracted from hippocampi of rats at the indicated time following induction of seizure by kainic acid injection.

Figure 2-7. CPG2 localizes to synapses in cultured hippocampal neurons

(A) Cultured hippocampal neuron (24 DIV) labeled with the anti-CPG2 monoclonal antibody 200A6.1. Scale bar: 10 μm. (B) Dendrite from a cultured hippocampal neuron infected with the *cpg2-gfp* lentivirus and stained for CPG2. (C) Cultured hippocampal neuron filled with GFP by lentivirus infection and stained for CPG2 and synapsin. Arrow indicates a synapse not labeled for CPG2. Scale bar: 2 μm.

Figure 2-8. CPG2 localizes to excitatory synapses

Cultured hippocampal neurons were filled with GFP by lentivirus infection and stained for CPG2 and (A) GAD65, a marker for inhibitory synapses, or (B) PSD-95, a postsynaptic marker for excitatory synapses. Arrow indicates a CPG2-positive shaft synapse. Scale bar: 2 μm.

Figure 2-9. CPG2 localizes to a subdomain of excitatory synapses

(A) High magnification images of individual spines from cultured hippocampal neurons labeled for actin filaments (green), PSD-95 (blue), and CPG2 (red). Scale bar: 1 μm. (B) Anti-CPG2 preembedding immunoEM of rat dentate gyrus showing CPG2 staining (arrow) in dendritic spines (left, middle) and shaft (right) synapses. Scale bar: 100 nm. (C) Anti-CPG2 preembedding immunoEM of cultured hippocampal neurons showing CPG2 staining in dendritic spine (left) and shaft (right) synapses. Scale bar: 100 nm. (D, E) Postembedding immonogold labeling of CPG2 and NR1 from adjacent serial section of the same dendritic spines from rat dentate gyrus. Scale bar: 100 nm. S = Spine, P = PSD, T = presynaptic terminal.

Figure 2-10. CPG2 localizes to an endocytic zone of excitatory synapses

(A) Cultured hippocampal neuron infected with a lentivirus expressing the clathrin light chain A1-GFP fusion protein and stained for CPG2 and PSD-95. Scale bar: 5 μm. (B) High-magnification views of individual spines from neurons as in A. Scale bar: 1 μm. Preembedding anti-CPG2 immunoEM showing CPG2 localization (arrows) near coated pits (asterisks) from (C) normal cultured hippocampal neurons and (D) hippocampal neurons treated with the actin depolymerizing agent latrunculin A. Scale bars: 100 nm. T = presynaptic terminal; P = PSD.

Chapter 3

CPG2 interacts with the actin-cytoskeleton and endophilin B2

Abstract

cpg2 is an activity-regulated transcript that encodes a protein specifically localized to the postsynaptic endocytic zone of excitatory synapses, indicating a potential function in regulating postsynaptic endocytosis. Since it contains only protein-protein interaction domains, CPG2 is likely to function as a scaffolding protein, potentially organizing proteins that regulate postsynaptic endocytosis. In this chapter, I discuss experiments performed to identify CPG2 interacting proteins and show that CPG2 interacts with itself, the actin cytoskeleton, and endophilin B2, a member of a family of membrane trafficking proteins. Each of these interactions is mediated by the CPG2 C-terminus that contains several coiled-coils motifs. Thus, CPG2 likely underlies a network of proteins at the postsynaptic endocytic zone that may be involved in the endocytosis of postsynaptic proteins.

Introduction

cpg2 is an activity-regulated regulated splice variant of the *syne-1* gene whose expression is limited to brain regions capable of synaptic plasticity. It encodes a protein that is localized specifically to the endocytic zone of excitatory synapses, where it associates with clathrin-coated pits. The postsynaptic endocytic zone is an integral synaptic component present in excitatory synapses, segregated from the postsynaptic density (Blanpied et al., 2002). It is the likely location of the internalization of glutamate receptors, an important mechanism for maintaining and altering synaptic strength that likely occurs through clathrin-mediated endocytosis (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Man et al., 2000; Snyder et al., 2001). CPG2's localization to this endocytic zone suggests that it may be a neuron-specific regulator of the endocytosis of postsynaptic proteins, including glutamate receptors.

The CPG2 protein contains solely sequences indicative of a structural function, including two spectrin repeats and several coiled-coil motifs. Coiled-coils are alpha-helical domains with a seven residue periodicity in which apolar residues reside in the fourth and seventh position, generating a hydrophobic surface of the helix (Burkhard et al., 2001). Often, they mediate protein homo- and heterodimerization via interaction of these hydrophobic surfaces (Burkhard et al., 2001). Spectrin repeats consist of a bundle of three alpha helices and, historically, have been viewed as modular structures that lengthen proteins and separate terminal domains (Djinovic-Carugo et al., 2002). However, there is evidence that spectrin repeats may serve to anchor networks of cytoskeletal, signal transduction, and transmembrane proteins (Djinovic-Carugo et al., 2002).

In this chapter, I describe my experiments designed to uncover CPG2-interacting proteins and show that it binds to itself and to the actin cytoskeleton, but indirectly to actin filaments. Furthermore, CPG2 interacts with the endocytic protein endophilin B2 and partially colocalizes with it within dendritic spines. The coiled-coil motifs within the CPG2 C-terminus likely mediate these interactions. Thus, CPG2 underlies the organization of a protein network at the postsynaptic endocytic zone that includes the actin cytoskeleton and endophilin B2, through which it may regulate the internalization of postsynaptic proteins.

Results

Proteins that contain coiled-coil domains often form dimers via interaction of their coiled-coils (Burkhard et al., 2001). Since CPG2 contains several predicted coiled-coils, a yeast two-hybrid assay was used to determine if CPG2 interacts with itself. CPG2 was subcloned into both the DNA-binding domain (DNA-BD) and the activation domain (AD) yeast two-hybrid plasmids. When CPG2-DNA-BD and CPG2-AD were cotransformed into the yeast strain AH109, yeast colonies grew on –Ade/-His/-Leu/-Trp selective plates (Figure 3-1), suggesting that CPG2 can self-associate and may form multimers. To define the domain of CPG2 that self-associates, truncation mutants of *cpg2* were subcloned into the DNA-BD plasmid and cotransformed with the full-length CPG2 in the AD plasmid into AH109 yeast. All truncation mutants that contained the final 135 amino acids of CPG2 allowed for yeast growth on selective plates, indicating an interaction with the full-length CPG2 (Figure 3-1). The mutants lacking this region did

not interact with the full-length CPG2. Thus, the C-terminus of CPG2, which contains several coiled-coils, is essential for its self-association.

Structural proteins that contain spectrin repeats and coiled-coils often interact with the actin cytoskeleton and anchor protein complexes to the cytoskeleton (Djinovic-Carugo et al., 2002). Although CPG2 does not contain the actin-binding domain of the Syne-1 protein, its structural motifs may contain actin binding regions. To determine if CPG2 interacts with actin filaments, an actin filament cosedimentation assay was performed. Actin forms filaments in a high salt buffer *in vitro*, which can be pelleted by ultracentrifugation, along with any interacting proteins. A preparation of highly purified (>99%) alpha-actin was added to either a low salt, non-polymerizing buffer or a high salt, polymerizing buffer. Radiolabeled CPG2 and luciferase (negative control) were added to the actin preparations, and actin filaments were pelleted. Proteins from the pellet and supernatant fractions were resolved on an SDS-PAGE gel. Both CPG2 and luciferase remained in the supernatant in both the low and high salt conditions and did not pellet with actin filaments (Figure 3-2, left), suggesting that CPG2 does not interact directly with actin filaments *in vitro*. Several proteins that interact with the actin cytoskeleton but not directly with actin filaments, including Arc (Lyford et al., 1995), do not pellet with highly purified actin, but do pellet with a less purified actin preparation that contains several proteins tightly associated with actin filaments. Therefore, the actin cosedimentation assay was repeated with a less purified actin preparation. CPG2 pelleted with actin filaments generated from this actin preparation (Figure 3-2, right). Thus, while CPG2 does not interact directly with actin filaments, it does interact with the actin

cytoskeleton through at least one mediator protein that remains in the less purified actin preparation.

The truncated mutants from the two hybrid assay were used in the actin cosedimentation assay to define the domain of CPG2 that interacts with the actin cytoskeleton. When the C-terminal and the N-terminal portions of CPG2 were added to the actin preparations, only the C-terminus pelleted with actin filaments (Figure 3-3A). When further truncations of the CPG2-C-terminus were added to the actin preparations, only CPG2 fragments that contained the C-terminal 135 amino acids cosedimented with actin filaments (Figure 3-3B). Thus, the CPG2 C-terminus is sufficient and necessary for both self-association and for CPG2's association with the actin cytoskeleton.

Since CPG2 interacts with the actin cytoskeleton, it may function to anchor specific proteins to the cytoskeleton. To isolate CPG2 interacting proteins, CPG2 was immunoprecipitated from rat brain with the anti-CPG2 monclonal antibody 200A5 and the immunoprecipitated proteins were silver stained to visualize proteins. A ~55 kD protein coimmunoprecipitated with CPG2 but not with the control antibody (Figure 3-4A). The band was excised and microsequenced via mass spectrometry. Several peptides sequenced from the excised band identified mouse endophilin B2. The mouse endophilin B2 full-length sequence was obtained and subcloned into an expression vector with a Flag-epitope tag and cotransfected with an HA-tagged *cpg2* into 293T cells. Protein extracts were immunoprecipated with an anti-Flag or anti-HA antibody. Flag-endophilin B2 coimmunoprecipitated with HA-CPG2 (Figure 3-4B), and HA-CPG2 coimmunoprecipitated with Flag-endophilin B2 from 293T cells (Figure 3-4C). Thus, CPG2 interacts with endophilin B2 when exogenously expressed in 293T cells. The

endophilin B2 sequence used in these experiments encoded a 44 kD protein, smaller than the protein identified in the immunoprecipitation. However, it has since been determined that this clone is a splice variant of a transcript that encodes a 54 kD protein (Accession #XM_231127), the proper size to be the protein that co-immunoprecipitates with CPG2 from rat brain.

All the endophilin family members, including endophilin B2, contain an N-terminal enzymatic domain, a dimerization domain that contains a coiled-coil motif, and a C-terminal SH3 domain for protein interactions (Figure 3-5A) (Huttner and Schmidt, 2000). Flag-tagged endophilin B2 mutants truncated to contain (EndoB2-C+DD) or omit (EndoB2-CΔDD) the dimerization domain were generated and cotransfected with CPG2 into 293T cells. EndoB2-C+DD coimmunoprecipitated with CPG2, while EndoB2-CΔDD did not, suggesting that the endophilin B2 dimerization domain mediates its interaction with CPG2 (Figure 3-5B). To determine the region of CPG2 that interacts with endophilin B2, the HA-tagged CPG2 truncation mutants were cotransfected with Flag-endophilin B2 into 293T cells and were immunoprecipitated with an anti-HA antibody. Endophilin B2 immunoprecipitated with each CPG2 fragment, suggesting that it can interact with different regions of CPG2. However, the CPG2 C-terminus immunoprecipitated endophilin B2 much more effectively than did the other CPG2 fragments (Figure 3-5C). These data suggest that, while all regions of CPG2 are capable of interacting with endophilin B2, the CPG2 C-terminus binds tightly to endophilin B2 in the absence of the other protein domains, which may serve to inhibit that binding in the full-length protein.

If CPG2 and enophilin B2 form a meaningful interaction in neurons, they would colocalize in excitatory synapses. To this end, a plasmid encoding an endophilin B2-GFP fusion protein was cotransfected with HA-*cpg2* into cultured hippocampal neurons, and the neurons were stained for CPG2. Endophilin B2-GFP formed a punctate pattern that colocalized with HA-CPG2, indicting a postsynaptic localization (Figure 3-5A). An anti-endophilin B2 polyclonal antibody was generated to identify the localization of the endogenous protein in neurons. Cultured neurons stained with this antibody and the anti-CPG2 antibody showed a punctate staining pattern consistent with a localization of endophilin B2 to dendritic spines (Figure 3-5B). This endophilin B2 staining partially colocalized with CPG2. These data suggest that CPG2 indeed interacts with endophilin B2 within dendritic spines.

Discussion

In the previous chapter, I showed that the activity-regulated transcript *cpg2* is a brain-specific splice variant of the *syne-1* gene that encodes a protein that localizes to the postsynaptic endocytic zone of excitatory synapses. In this chapter, I show that, mediated by its C-terminus, CPG2 interacts with itself, the actin cytoskeleton, and the endocytic protein endophilin B2. Thus, consistent with its predicted structure, CPG2 appears to organize a complex of proteins at the postsynaptic endocytic zone.

CPG2 interacts with itself and the actin cytoskeleton

CPG2 contains several amino acid segments, mainly concentrated in the C-terminus, predicted to contain coiled-coil motifs, which often underlie the formation of dimers (Burkhard et al., 2001). The hydrophobic residues exposed on the surface of the alpha-

helices of the coils of separate proteins bind to one another in either a parallel or antiparallel direction (Burkhard et al., 2001). The C-terminus of CPG2 contains three predicted coiled-coils and interacts with the full-length CPG2 protein in a two-hybrid assay, suggesting that the coiled-coils of CPG2 can indeed mediate protein dimerization. It is unclear whether CPG2 forms dimers *in vivo*.

Most members of the spectrin family of proteins interact with actin filaments, normally through an actin binding domain, and use this interaction to anchor other proteins to the actin cytoskeleton (Djinovic-Carugo et al., 2002). For example, in excitatory synapses, spectrin and alpha-actinin both contain an actin binding domain that may mediate the anchorage of NMDA receptors to the actin cytoskeleton of dendritic spines (Jancsik and Hajos, 1998; Lidov et al., 1990; Wechsler and Teichberg, 1998; Wyszynski et al., 1997). Not surprisingly, given it does not contain the Syne-1 actin-binding domain, CPG2 does not interact directly with actin filaments. However, CPG2 does interact with the actin cytoskeleton. Like several other proteins that are known to interact with the actin cytoskeleton but not directly to actin filaments, CPG2 cosediments with actin filaments generated from a less purified actin preparation that contains proteins tightly associated with actin filaments. Thus, CPG2 presumably binds to at least one protein remaining in the actin preparation that mediates an interaction with actin filaments. The identity of this mediator protein(s) is unclear. However, these data suggest that CPG2, like other spectrin family proteins, may play a role in regulating the anchorage of a set of interacting proteins to the actin cytoskeleton. Unlike other spectrin family members, this interaction is indirect and is not mediated by an actin binding domain.

CPG2 interacts with endophilin B2

Mass spectrometry microsequencing of a ~55 kD protein that coimmunoprecipitates with CPG2 identified endophilin B2 as a CPG2 interacting protein. CPG2 and endophilin B2 coimmunoprecipitate each other from 293T cells. Furthermore, transfected GFP-tagged endophilin B2 colocalizes with HA-CPG2 in dendritic spines in hippocampal neurons, and endogenous endophilin B2 antibody staining partially overlaps with CPG2 staining. These data suggest that CPG2 may indeed interact with endophilin B2 at excitatory synapses in hippocampal neurons.

All members of the endophilin family of proteins have a similar domain structure, consisting of an N-terminal enzymatic domain, a dimerization domain, and a C-terminal SH3 domain for mediating protein interactions (Huttner and Schmidt, 2000). Endophilin A1, the most extensively studied member of the endophilin family of proteins, is localized to the presynaptic terminal, where it plays a critical role in synaptic vesicle endocytosis (Ringstad et al., 1999; Verstreken et al., 2002). Through its enzymatic domain, it is thought to regulate the lipid composition, and therefore the curvature, of invaginating synaptic vesicles by catalyzing a change from inverted cone-shaped phospholipids to cone-shaped phospholipids at the neck of pinching off vesicles (Farsad et al., 2001; Schmidt et al., 1999). In addition, recent evidence suggests that endophilin A1 may also act as an adapter protein, localizing other critical endocytic proteins, largely through its SH3 domain, to internalized synaptic vesicles (Fabian-Fine et al., 2003; Verstreken et al., 2003). In the *Drosophila* neuromuscular junction, endophilin A1 mutants result in the accumulation of clathrin-coated vesicles, likely due to the disruption of synaptojanin targeting to the synapse (Verstreken et al., 2003). Synaptojanin is

essential for the removal of the clathrin coats from vesicles and for their reinsertion into the presynaptic vesicle cycle (Gad et al., 2000). The endophilin Bs contain a similar domain structure, and endophilin B1, like endophilin A1, can tubulate liposomes, suggesting it can regulate lipid structure (Farsad et al., 2001; Modregger et al., 2003; Pierrat et al., 2001). However, endophilin B1b does not localize to synaptic terminals and is thought to play a role in membrane trafficking from intracellular membrane compartments (Modregger et al., 2003). Thus, it is not surprising that endophilin B2 localizes not to presynaptic terminals, but to dendritic spines, where it may regulate the trafficking of membrane proteins into and out of the postsynaptic membrane. The function of the interaction between CPG2 and endophilin B2 is unknown, but I speculate that CPG2, like other spectrin family members, may organize and anchor a set of signaling and/or structural proteins. Importantly, this interaction is mediated by the dimerization domain of endophilin B2, which leaves its SH3 domain free for protein interactions. Since endophilin A1 is thought to be, in part, an adapter protein linking critical endocytic proteins (Fabian-Fine et al., 2003; Verstreken et al., 2003), endophilin B2 may play a similar role, and CPG2 may regulate this protein complex at postsynaptic endocytic sites.

Each of these protein interactions is mediated by the C-terminus of CPG2, raising the possibility that this region of CPG2 is the most critical for its function. This idea is supported by the likelihood that translation initiation of *cpg2* is leaky, resulting in a protein truncated at the N-terminus. If the protein interactions of the CPG2 C-terminus are critical for its function, then these the truncated protein should act identically as the full-length. Additionally, it is possible that *cpg2b* encodes the same proteins as *cpg2* due

to leaky translation initiation, as there is no protein band in Western blots corresponding to the predicted CPG2B protein. The importance of the CPG2 C-terminus may explain the need for two such closely related splice-variants, as having two activity-regulated transcription initiation sites would ensure its expression.

Methods

Two-hybrid assay

The full-length *cpg2* cDNA and the truncated mutants, amplified via PCR, were subcloned into the DNA-binding domain vector (DNA-BD; pGBKT7), and the *cpg2* cDNA was subcloned into the activation domain vector (AD; pGADT7) of the Matchmkaker 3 Yeast Two-Hybrid System (Clontech). The *cpg2*-AD and *cpg2*-DNA-BD constructs were transformed into yeast strain AH109 as follows. YPDA yeast medium was inoculated with an overnight culture of AH109 and shaken at 30°C overnight. Cells were centrifuged for 5 minute at 1000 RPM, resuspended in sterile water, centrifuged again, and resuspended in 1X TE/LiAc (1X TE and 1 M LiAc). 1 μg of both the AD and DNA-BD plasmids and 20 μl of herring testes carrier DNA (Clontech) were mixed, and 0.2 ml of yeast mixture and 1 ml of sterile PEG/LiAc (1X TE, 1 M LiAc, 40% polyethylene glycol) were added. Yeast were incubated at 30°C for 30 min and then heat shocked for 15 min at 42°C. Yeast were chilled on ice, centrifuged at 14000 RPM for 30 sec, resuspended in sterile water, and plated onto -Leu/-Trp plates. After 3 days, colonies positive for both plasmids were streaked onto -Ade/-His/-Leu/-Trp plates. Growth was checked after 3 days of incubation at 30°C.

Actin co-sedimentation assay

[^{35}S]-methionine labeled proteins were generated *in vitro* with the TNT Quick-Coupled Transcription/Translation System (Promega). Transcription from the T7 promoter of the *cpg2* full-length cDNA in pBluescript II (Stratagene), the *cpg2* truncated mutants in pGBKT7 (Clontech), and luciferase (supplied with the kit) were performed according to the manufacturer's protocol. For actin co-sedimentation, 250 μg of >99% pure α-actin (Cytoskeleton) or 90% pure α-actin (Sigma) were resuspended to 0.5 mg/ml in general actin buffer (5 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM DTT). 90 μl of this solution was placed into two separate tubes, and 10 μl of 10X actin polymerization buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) was added to the high salt tube to induce polymerization of actin, while 10 μl of general actin buffer was added to the low salt tube. Tubes were incubated at room temperature for 1 hour. Following actin polymerization, 2 μl of [^{35}S]-methionine labeled CPG2 or CPG2 fragments and 2 μl of [^{35}S]-methionine labeled luciferase were added. Tubes were incubated for 30 minutes at room temperature to allow actin binding and then centrifuged at 150000xg for 1.5 hours at room temperature to pellet actin filaments. Supernatants were removed and 20 μl of 5X SDS-PAGE loading buffer was added. Pellets were resuspended in 60 μl of 1X SDS-PAGE loading buffer. Samples were boiled, and 20 μl of each were run on a 9% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane, and bands were visualized by autradiography overnight at -70°C.

Immunoprecipitations and mass spectrometry

For tissue immunoprecipitations, cerebral cortices and hippocampi of adult rats were homogenized in NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% NP-40) plus a protease inhibitor cocktail (1:200; Sigma) with 10 strokes in a Teflon-glass homogenizer. Cellular debris and insoluble proteins were pelleted via centrifugation at 14000 RPM at 4°C. Supernatants were collected, 5 μg of anti-CPG2 monoclonal antibody 200A5 or mouse IgG (Sigma) was added, and tubes were incubated overnight at 4°C with gentle rocking. Protein A-agarose slurry (20 μl; Sigma) was added and incubated for 2 hours. Immune complexes were pelleted, washed 3 times in NP-40 buffer, and resuspended in SDS-PAGE loading buffer. After boiling for 5 minutes, samples were separated on an 8% SDS-PAGE gel and silver stained with the GelCode SilverSnap Stain Kit (Pierce). For mass spectrometry, the cortices and hippocampi of 8 adult rats were immunoprecipitated with the anti-CPG2 monoclonal antibody 200A5, and immune complexes were separated on an 8% SDS-PAGE gel. The gel was stained with Coomasie blue, and the 55 kD band was excised and placed into a microcentrifuge tube. Gel fragments were rinsed three times in acetonitrile, frozen, and sent to the Harvard Microchemistry Facility for mass spectrometry analysis.

For 293T cell immunoprecipitations, mouse endophilin B2 was amplified from an endophilin B2-GFP fusion construct (a gift of Dr. Nan-Shan Chang) and subcloned into pcDNA3 (Promega) with a Flag epitope tag. Flag-endophilin B2 or EGFP-C1 was cotransfected with HA-*cpg2* or EGFP-C1 into 293T cells with Lipofectamine2000 (Invitrogen). After 24 hours, cells were rinsed twice in PBS and lysed with NP-40 buffer. Protein extracts were collected into microcentrifuge tubes and centrifuged at 14000 RPM for 15 min at 4°C. Immunoprecipitations were done as described with 5 μg of mouse

anti-Flag antibody (Sigma) or rabbit anti-HA antibody (Santa Cruz). Proteins were separated on an 8% SDS-PAGE gel, transferred to nitrocellulose, probed with the rabbit anti-CPG2 polyclonal antiserum (1:5000) or mouse anti-Flag antibody (1:1000), and developed with ECL (Amersham). Truncated *cpg2* and endophilin B2 mutants were generated by PCR and subcloned into pcDNA3 including an HA or Flag epitope, respectively, and immunoprecipitations from 293T cells were performed as described.

Hippocampal cultures

Hippocampal cultures were made essentially as described (Brewer et al., 1993). Glass coverslips (Carolina Biological Supply) in culture dishes were incubated in poly-D-lysine (40 μg/ml) and laminin (2.5 μg/ml; BD Biosciences) overnight, rinsed 3 times with water, and incubated in plating medium: Neurobasal (Gibco) supplemented with 0.5 mM L-glutamine, 12.5 μM L-glutamate, and B27 (Gibco). Hippocampi from E18 embryos were isolated, digested in 0.25% trypsin (Gibco) plus 0.1% DNAse (Sigma), and triturated with fire-polished pipettes in 0.1% DNAse. Cells were then centrifuged at 1000 RPM for 10 min at 4°C, resuspended in plating medium, counted, and plated at $1.6X10^4$ cells/cm^2. After 4 days, one half of the medium was replaced with plating medium without additional L-glutamate.

Immunocytochemistry and fluorescent microscopy

Rabbit anti-endophilin B2 antibody was generated against an endophilin B2 specific peptide (DPDWLIGERGNKKGK) and purified with that peptide (Open Biosystems). Cells were fixed in 4% formaldehyde at 4°C for 20 min, permeabilized in 0.3% Triton-X-100/PBS for 5 min at 4°C, and blocked in 10% goat serum in PBS for 1 hour at 4°C.

Cultures were then incubated overnight in primary antibodies in PBS at 4°C, rinsed 3 times in PBS, and incubated in secondary antibodies for 1 hour at room temperature. Following 3 washes in PBS, cultures were mounted onto coverslips with Flouromout G (Southern Biotechnology). Primary antibodies were as follows: mouse anti-CPG2 monoclonal 200A6.1 (1 μg/ml) and rabbit anti-endophilin B2 (0.2 μg/ml). Secondary antibodies were as follows: goat anti-rabbit Alexa Flour488 (1:500; Molecular Probes) and goat anti-mouse Alexa Flour555 (1:500; Molecular Probes). Flourescent images of neurons were imported into Spot 3.5 (Diagnostic Instruments) with a Diagnostic Instruments Spot2 digital camera mounted on a Nikon Eclipse E600 using a 60X/1.40 Plan Apo oil immersion objective (Nikon). Images were saved as gray-scale TIFFs and overlayed and pseudo-colored for figures with Photoshop5.0. For endophilin B2-GFP transfections, hippocampal cultures (20DIV) were transfected with endophilin B2-GFP and HA-*cpg2* using Lipofectamine2000 (Invitrogen). After 24 hours, cells were fixed, stained with the anti-CPG2 antibody, and mounted onto coverslips.

Figure 3-1. CPG2 interacts with its C-terminus

Name	Residues	Schematic	Growth
CPG2	941		+
CPG2-N	402		-
CPG2-C	539		+
CPG2-C.1	269		-
CPG2-C.2	269		+
CPG2-C.2.2	135		+

Figure 3-2. CPG2 interacts with the actin cytoskeleton

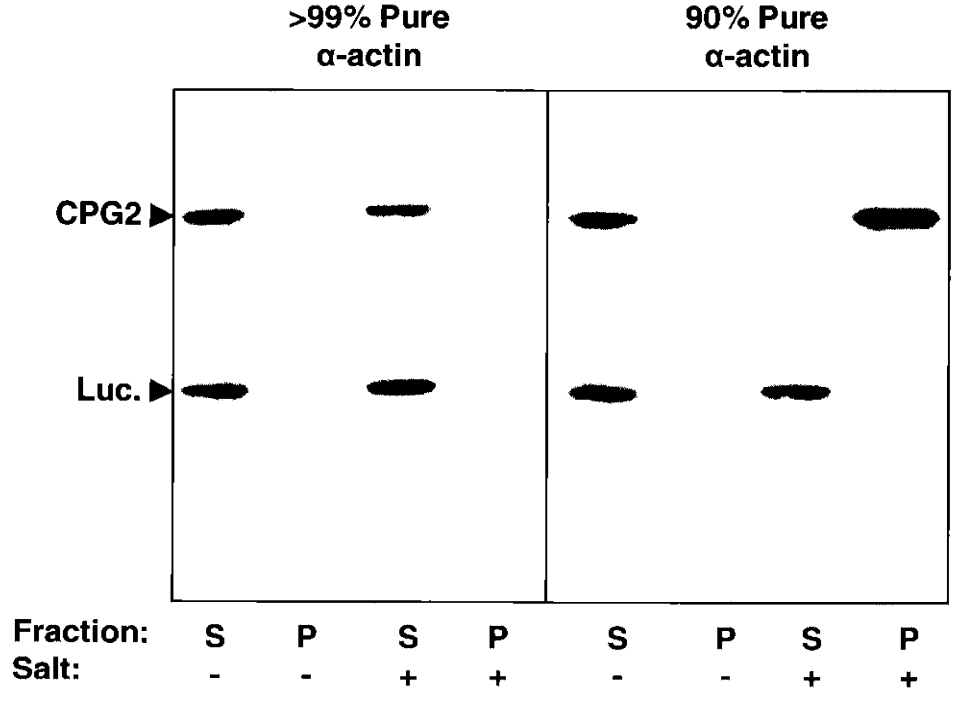

Figure 3-3. CPG2 C-terminus interacts with the actin cytoskeleton

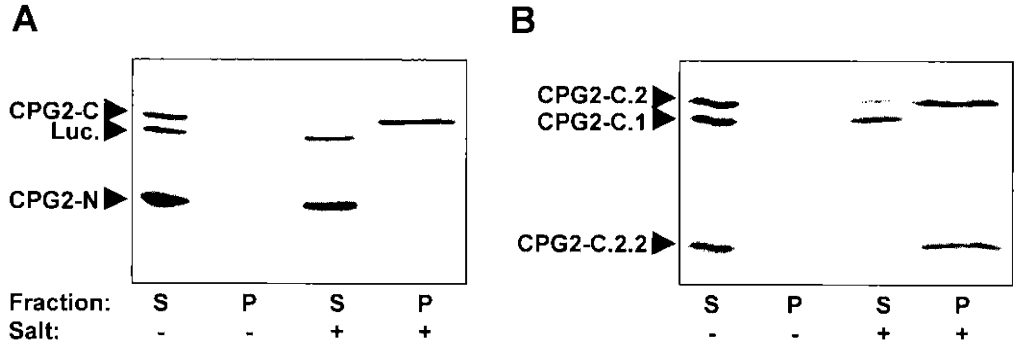

Figure 3-4. CPG2 interacts with endophilin B2

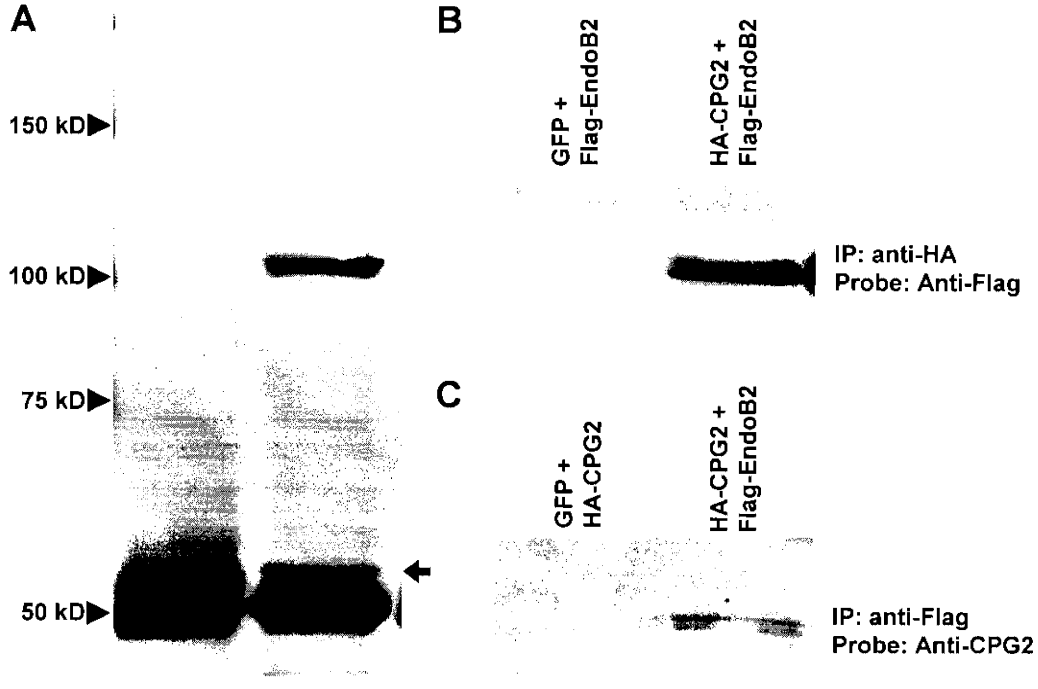

Figure 3-5. CPG2-endophilin B2 domain interactions

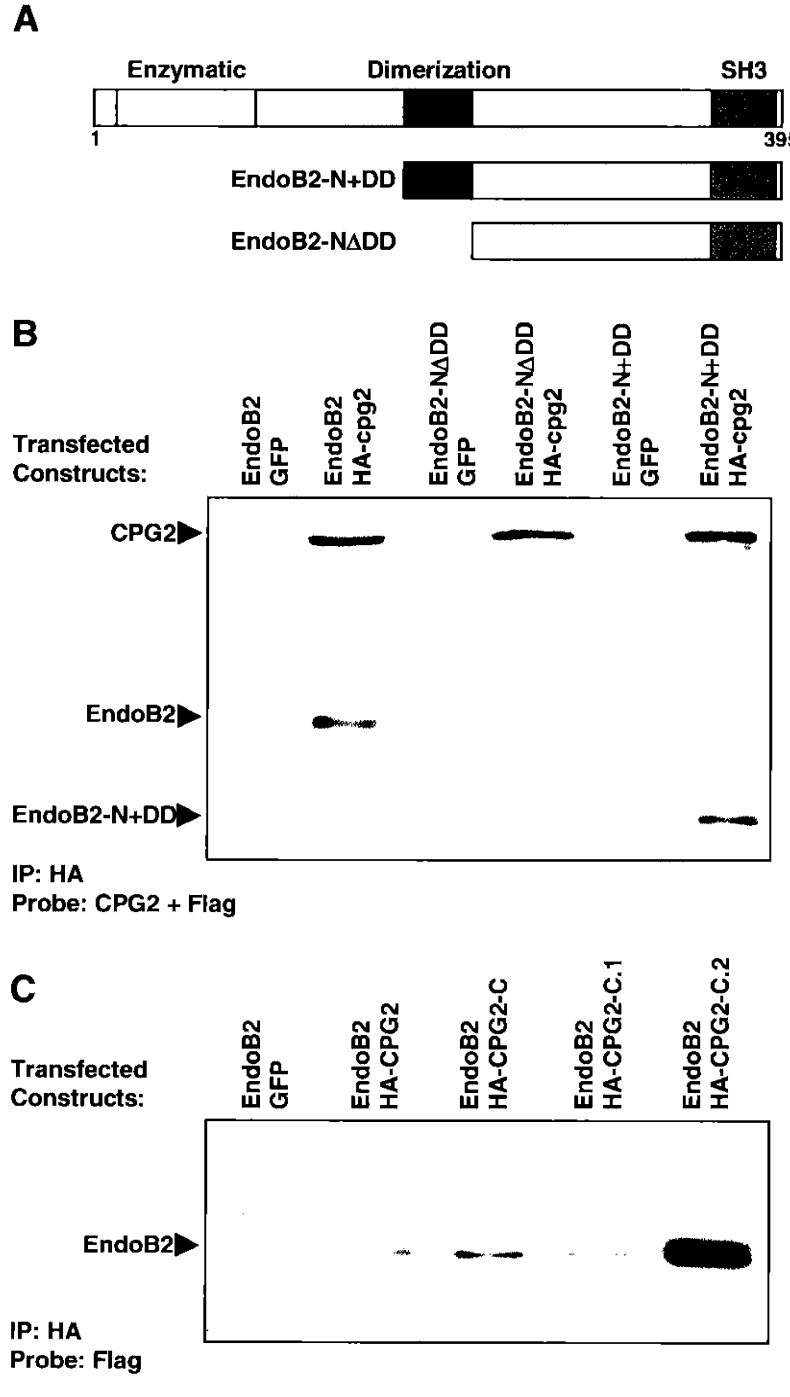

Figure 3-6. CPG2 partially colocalizes with endophilin B2 in dendritic spines

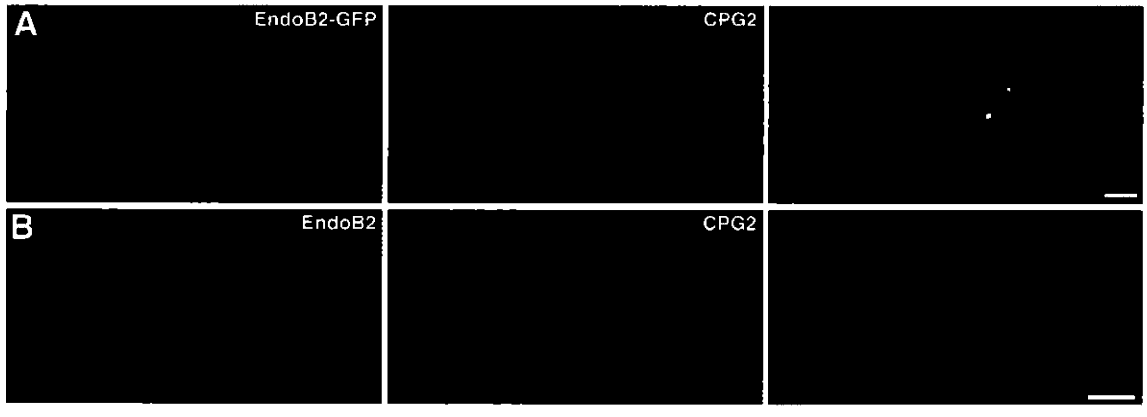

Figure 3-1. CPG2 interacts with its C-terminus

CPG2 fragments were subcloned into the pGBKT7 DNA-BD plasmid, co-transformed with CPG2 subcloned into the pGADT7 AD plasmid into yeast strain AH109, and plated onto –Ade/-His/-Leu/-Trp selective plates. Righthand column indicates growth of yeast colonies on these plates. Schematics indicate CPG2 fragments. Blue boxes show locations of predicted coiled-coils.

Figure 3-2. CPG2 interacts with the actin cytoskeleton

Actin cosedimentation assays were performed with either highly purified actin (left) or a less purfied actin preparation (right) and [^{35}S]-methionine labeled CPG2 and luciferase (negative control). Luc. = Luciferase; S = supernatant; P = pellet; - = low salt; + = high salt, polymerizing buffer. CPG2 pellets with less purified actin filament preparation in the high salt condition.

Figure 3-3. CPG2 C-terminus interacts with actin cytoskeleton

(A,B) Actin cosedimentation using less purified actin preparation and [^{35}S]-methionine labeled CPG2 truncated mutants shown in Figure 3-1. Luc. = Luciferase; S = supernatant; P = pellet; - = low salt; + = high salt. The CPG2 mutant containing the 135 amino acids at the C-terminus pelleted with less purified actin preparation in the high salt condition.

Figure 3-4. CPG2 interacts with endophilin B2

(A) Silver stain of coimmunoprecipitations from rat brain using control mouse IgG (left lane) or anti-CPG2 monoclonal antibody 200A5 (right lane). Note CPG2 at 110 kD and the additional band at ~55kD (arrow). (B) Coimmunoprecipitation of Flag-tagged endophilin B2 with HA-tagged CPG2 from 293T cells. (C) Coimmunoprecipitation of HA-tagged CPG2 with Flag-tagged endophilin B2 from 293T cells.

Figure 3-5. CPG2-endophilin B2 domain interactions

(A) Domain map of endophilin B2 and schematic of the Flag-tagged truncation mutants. (B) 293T cells cotransfected with the indicated plasmids were immunprecipitated with the anti-HA antibody and probed with the anti-Flag and anti-CPG2 antibodies. (C) 293T cells cotransfected with Flag-endophilin B2 and the indicated plasmids were immunoprecipitated with the anti-HA antibody and probed with the anti-Flag antibody.

Figure 3-6. CPG2 partially colocalizes with endophilin B2 in dendritic spines

(A) GFP-endophilin B2 and HA-*cpg2* were cotransfected into cultured hippocampal neurons and stained for CPG2. Scale bar: 3 μm. (B) Cultured hippocampal neurons were stained for endogenous endophilin B2 and CPG2. Scale bar: 2 μm.

Chapter 4

CPG2 is an essential component of the glutamate receptor endocytic pathway

Abstract

CPG2 is the product of a brain-specific, activity-regulated transcript and localizes to the postsynaptic endocytic zone of excitatory synapses, where it likely organizes a network of proteins, including the endocytic protein endophilin B2. These data suggest that CPG2 may play an important role in the endocytosis of synaptic proteins, including glutamate receptors. In this chapter, I manipulate CPG2 levels using RNA interference (RNAi) and overexpression to illuminate the function of CPG2 within synapses. CPG2 knock-down increases the number of postsynaptic clathrin-coated vesicles, some of which traffic NMDA receptors, and disrupts the internalization of AMPA and NMDA receptors. Furthermore, CPG2 levels affect dendritic spine size, consistent with a role in regulating membrane trafficking. Thus, CPG2 is a critical component of the glutamate receptor endocytic pathway. As a brain-specific protein, CPG2 may be an essential organizer of a novel specialization that mediates glutamate receptor endocytosis.

Introduction

Modification of the number of synaptic glutamate receptors is a primary mechanism for regulating synaptic strength (Malinow and Malenka, 2002). Glutamate receptors undergo constitutive internalization. For AMPA receptors, this internalization is rapid and regulated by synaptic activity (Ehlers, 2000; Lin et al., 2000). For NMDA receptors, internalization in mature neurons is slower and not regulated by activity, but is rapid in immature neurons (Ehlers, 2000; Lin et al., 2000; Roche et al., 2001). Long-term depression (LTD) likely results, in part, from the internalization of synaptic glutamate receptors. A number of treatments that induce LTD also induce the internalization of AMPA receptors (Beattie et al., 2000; Lin et al., 2000; Man et al., 2000; Snyder et al., 2001). Although synaptic NMDA receptors are thought to be relatively stable, certain stimuli can induce their internalization (Nong et al., 2003; Snyder et al., 2001).

Glutamate receptor internalization likely occurs through clathrin-mediated endocytosis (Beattie et al., 2000; Carroll et al., 1999; Ehlers, 2000; Lin et al., 2000; Man et al., 2000), a general mechanism for the internalization of proteins from the plasma membrane (Mousavi et al., 2004). Clathrin-mediated endocytosis occurs at stable, persistent endocytic regions on the membrane of heterologous cells (Gaidarov et al., 1999; Santini et al., 2002; Scott et al., 2002). A similar endocytic zone, spatially segregated from the postsynaptic density (PSD), has recently been shown to localize to the postsynaptic side of excitatory synapses and may control the internalization of synaptic proteins, including glutamate receptors (Blanpied et al., 2002). However, the mechanisms of synaptic glutamate receptor internalization are not well understood. Although they are likely internalized via clathrin-mediated endocytosis, glutamate

receptors have never been observed in clathrin-coated pits or vesicles at postsynaptic sites. In addition, there is no evidence that neurons have developed novel mechanisms for specifically regulating the endocytosis of glutamate receptors at the postsynaptic site.

In the previous chapters, I have shown that the *cpg2* transcript is an activity-regulated, brain-specific splice-variant of the *syne-1* gene and encodes a protein that localizes to the endocytic zone of excitatory synapses. Analysis of CPG2 interacting proteins showed that it likely forms a complex with the actin cytoskeleton and the endocytic protein endophilin B2. Here, I present evidence that CPG2 is a critical component of the postsynaptic endocytic pathway that mediates glutamate receptor internalization. As the first known brain- and synapse-specific regulator of clathrin-mediated endocytosis, I hypothesize that CPG2 may be a component of a novel postsynaptic specialization devoted to the internalization of postsynaptic proteins, including glutamate receptors, at synapses capable of plasticity.

Results

The localization of CPG2 to the postsynaptic endocytic zone and its association with endophilin B2 suggests that it may regulate the internalization of postsynaptic proteins. To test this hypothesis, I manipulated CPG2 protein levels using overexpression to increase and RNA interference (RNAi) to decrease CPG2 levels and examined the effects on spine morphology, clathrin-coated pit trafficking, and glutamate receptor internalization.

For CPG2 overexpression, a lentivirus was generated that results in the expression of a CPG2-GFP fusion protein driven by the human ubiquitin-C promoter (*cpg2-gfp*).

CPG2 levels were knocked-down with RNA interference using a small hairpin RNA (shRNA) specific to *cpg2*. To find a *cpg2* sequence that would be effective in RNAi, five separate DNA oligonucleotides encoding shRNA sequences specific to *cpg2* were subcloned into a plasmid downstream of the U6 promoter. This construct results in the expression of a shRNA that is cleaved by the Dicer complex to form a siRNA that is active for RNAi (Hutvagner and Zamore, 2002) (Figure 4-1A). These shRNA constructs were tested for RNAi by co-transfecting them with a *cpg2-IRES-GFP* construct into 293T cells. Since the *cpg2* and *GFP* sequences are contained on the same transcript, RNAi against *cpg2* also knocks-down GFP protein levels. One shRNA construct (*cpg2-shRNA*) resulted in >80% reduction in GFP and CPG2 protein levels as assayed by Western blotting (Figure 4-1B) and immunofluorescence (Figure 4-1C). Thus, exongenous CPG2 can be effectively knocked-down in 293T cells.

To induce RNAi-mediated knock-down of CPG2 in neurons, the *cpg2-shRNA* was delivered into cultured neurons via lentivirus infection. The *cpg2-shRNA* DNA sequence and the upstream U6 promoter were subcloned into the pFUGW lentivirus transfer vector upstream of the human ubiquitin-C promoter that drives expression of GFP (Figure 4-2A). Cells infected with this virus express both *cpg2-shRNA* and GFP, which marks infected cells and fills them for morphological analysis. A mutated version of the *cpg2-shRNA* (*Mcpg2-shRNA*) was generated in which five internal nucleotides were inverted. In a Western blot, high density cortical cultures infected with *cpg2-shRNA* showed a near elimination of CPG2 levels compared with *Mcpg2-shRNA* infected neurons (Figure 4-2B). While hippocampal neurons infected with the *Mcpg2-shRNA* showed a normal distribution of CPG2 at synaptic sites, *cpg2-shRNA* infected neurons showed little CPG2

staining within individual spines (Figure 4-2C). The distribution of PSD-95 and other synaptic proteins in *cpg2-shRNA* infected neurons showed no obvious changes in levels or distribution (Figure 4-2C and data not shown). Thus, endogenous CPG2 can be effectively and specifically knocked-down with the *cpg2-shRNA* lentivirus.

If CPG2 regulates the internalization of membrane from the spine surface, then alteration of CPG2 levels may affect the size of dendritic spines. Therefore, *cpg2-shRNA* and *cpg2-gfp* were used to assess the effects of CPG2 level changes on spine morphology. Cultured hippocampal neurons were infected with *Mcpg2-shRNA*, *cpg2-shRNA*, or *cpg2-gfp* at 4 DIV and fix and stained for CPG2 and PSD-95 at 24 DIV. Figure 4-3A shows examples of spines from neurons from each group. The area of the spine heads were measured in neurons from all groups. Knock-down of CPG2 levels resulted in a significant reduction in spine head area (-18% ± 2.4%, $p < 0.0005$), while CPG2 overexpression resulted in a significant increase in spine head area (+7% ± 2.1%; $p < 0.05$; n = 10 cells/group, ~100 spines/cell) relative to spines from *Mcpg2-shRNA* neurons (Figure 4-3B, C). Thus, CPG2 levels affect dendritic spine size, possibly by controlling membrane trafficking through endocytosis.

To test this possibility, EM analysis of *cpg2-shRNA* infected neurons was performed to assess the number of synapse-associated clathrin coated pits and/or vesicles. Figure 4-4A shows examples of spine and shaft synapses with and without clathrin-coated vesicles from *Mcpg2-shRNA* and *cpg2-shRNA* infected neurons. Quantification of the number of synapse-associated clathrin coated pits and vesicles showed that there was a 4-fold increase in the number of these structures in *cpg2-shRNA* (0.95 ± 0.18 per synapse; n = 28 neurons) versus *Mcpg2-shRNA* (0.25 ± 0.06 per synapse; n = 35 neurons;

$p < 0.0002$) infected neurons (Figure 4-4B). Thus, CPG2 knock-down increases the number of postsynaptic clathrin-coated pits and vesicles in the vicinity of synapses.

To determine whether the clathrin-coated pits and vesicles that accumulate in response to CPG2 knock-down contain synaptic proteins, immunoEM for the NR1 subunit of the NMDA receptor was performed on *cpg2-shRNA* treated cultures Some clathrin-coated vesicles in the vicinity of synapses from CPG2-knock-down neurons were positively stained for NMDA receptors (Figure 4-4A, B), whereas there was no staining seen on similar vesicles on unstained control cultures (Figure 4-4C). These data show that the CPG2 knock-down increases the number of postsynaptic clathrin-coated vesicles, some of which traffic NMDA receptors. Thus, CPG2 may regulate the endocytosis of glutamate receptors.

A biochemical internalization assay was used to confirm a role for CPG2 in the endocytosis of glutamate receptors. Surface proteins from cortical cultures infected with *Mcpg2-shRNA* or *cpg2-shRNA* were biotinylated with a reversible biotin and kept at 4°C to block internalization or moved to 37°C for 30 minutes to allow for receptor internalization (in the presence of a proteasome inhibitor). Remaining surface biotin was stripped with a cell-impermeable cleavage buffer. Biotinylated proteins were then isolated with neutravidin, probed on a Western blot, and compared with calibration controls. In *Mcpg2-shRNA* infected cultures, $6.2 \pm 0.8\%$ of surface NMDA receptors and $8.2 \pm 0.6\%$ of surface AMPA receptors were internalized after 30 minutes at 37°C (Figure 7D, E), similar to what has been previously reported for glutamate receptor internalization in cultured neurons (Ehlers, 2000; Lin et al., 2000). In *cpg2-shRNA* infected cultures, AMPA and NMDA receptor internalization was reduced to $2.7 \pm 0.8\%$

($p < 0.005$; $n = 3$) and 1.7 ± 0.5% ($p < 0.0003$; $n = 4$), respectively (Figure 7D, E). In contrast, there was no significant change in the internalization of insulin receptors in the *cpg2-shRNA* treated neurons (4.3 ± 0.4%) versus *Mcpg2-shRNA* treated neurons (5.4 ± 0.8%; $n = 4$, $p = 0.4$) (Figure 7D, E), indicating that the *cpg2-shRNA* blockade was specific to the internalization of synaptic proteins. While insulin receptor internalization was only reduced by 17 ± 8% in *cpg2-shRNA* infected neurons, the internalization of AMPA and NMDA receptors was reduced by 66 ± 11% ($p < 0.02$) and 73 ± 5% ($p < 0.002$), respectively, in *cpg2-shRNA* infected neurons (Figure 7F). The reduction in glutamate receptor internalization was not a result of a general reduction in synaptic protein levels, as total GluR2 and NR1 levels remained constant in *Mcpg2-shRNA* and *cpg2-shRNA* infected neurons (Figure 7D, bottom). Furthermore, in a surface protein biotinylation assay, there was no reduction in the amount of surface AMPA or NMDA receptors in *cpg2-shRNA* infected neurons, showing that the deficit in glutamate receptor internalization does not result from a reduction in the number of inserted receptors (Figure 7G). Thus, CPG2 knock-down disrupts glutamate receptor internalization, suggesting that CPG2 is an important regulator of this process.

Discussion

In the previous chapters, I showed that CPG2 is the product of an activity-regulated, brain-specific splice-variant of *syne-1*. It localizes to the endocytic zone of excitatory synapses, where it likely organizes a complex of proteins, including the actin cytoskeleton and endophilin B2. In this chapter, I present data indicating that CPG2 is a critical regulator of endocytosis of glutamate receptors at excitatory synapses. Changes in

CPG2 levels resulted in changes in dendritic spine size, possibly due to changes in membrane trafficking. Furthermore, RNAi-mediated knock-down of CPG2 levels increased the number of postsynaptic clathrin-coated vesicles and disrupted glutamate receptor internalization.

CPG2 knock-down led to a phenotype similar to that seen in the presynaptic terminals of organisms with endophilin mutations, where there is an accumulation of clathrin-coated synaptic vesicles and a disruption of the vesicle cycle due to a deficit in a late step of the synaptic vesicle processing (Cremona et al., 1999; Harris et al., 2000; Kim et al., 2002; Verstreken et al., 2002; Verstreken et al., 2003).This similarity suggests that CPG2 is an important regulator of a late step in the endocytosis of glutamate receptors, and disrupting this step disrupts the entire internalization pathway. In Chapter 5, I propose a model for the function of CPG2 in glutamate receptor internalization in light of its specific expression pattern and its proposed binding partners.

In CPG2 knock-down neurons, surface expression of glutamate receptors is relatively normal despite the disruption of glutamate receptor endocytosis. While acute blockade of endocytosis was shown to cause an increase in synaptic transmission (Luscher et al., 1999), these results are consistent with other findings that endocytosis inhibition does not affect the number of surface AMPA receptors (Lledo et al., 1998; Man et al., 2000), likely due to a tight link between the rates of receptor insertion into and removal from the synaptic membrane (Ehlers, 2000; Liang and Huganir, 2001). Considering there is a decrease in glutamate receptor internalization in these neurons, there must be a compensatory decrease in glutamate receptor insertion for steady state surface receptors to remain constant. In addition, the long-term nature of the RNAi

treatment likely allows homeostatic mechanisms to achieve a steady state of surface glutamate receptor levels, as chronic changes in activity at the network and single cell level have been shown to result in compensatory changes in synaptic strength (Burrone et al., 2002; Turrigiano et al., 1998). Thus, CPG2 knock-down appears to slow not only the internalization, but also the insertion, of glutamate receptors, suggesting that CPG2 may be necessary for the rapid cycling of synaptic glutamate receptors.

Dendritic spines on CPG2 knock-down neurons are nearly twenty percent smaller than in control neurons, likely resulting from an increase in membrane retention within internalized clathrin-coated vesicles. Interestingly, while surface glutamate receptors maintained a steady state number, spine size did not, suggesting that there is no compensatory mechanism for regulating membrane insertion into dendritic spines. While normally there is a tight correlation between spine size and synaptic AMPA receptor numbers (Kasai et al., 2003), in CPG2 knock-down neurons, the total amount of surface glutamate receptors remained constant despite smaller spines. These data could be explained if CPG2 knock-down causes a decrease in synaptic receptors and either an increase in extrasynaptic receptor density or in total synapse number. Also, CPG2 knock-down could disrupt the spine size-receptor number relationship and result in an increase in synaptic receptor density. Electrophysiological analysis of CPG2 knock-down neurons would distinguish between these possibilities.

Methods

Hippocampal cultures

Hippocampal cultures were made essentially as described (Brewer et al., 1993). Glass coverslips (Carolina Biological Supply) in culture dishes were incubated in poly-D-lysine (40 μg/ml) and laminin (2.5 μg/ml; BD Biosciences) overnight, rinsed 3 times with water, and incubated in plating medium: Neurobasal (Gibco) supplemented with 0.5 mM L-glutamine, 12.5 μM L-glutamate, and B27 (Gibco). Hippocampi from E18 embryos were isolated, digested in 0.25% trypsin (Gibco) plus 0.1% DNAse (Sigma), and triturated with fire-polished pipettes in 0.1% DNAse. Cells were then centrifuged at 1000 RPM for 10 min at 4°C, resuspended in plating medium, counted, and plated at 1.6×10^4 cells/cm^2. After 4 days, one half of the medium was replaced with plating medium without the addition of L-glutamate. Postnatal high-density cortical cultures were generated as described above, with the following changes. Cortices from P1 rats were digested, dissociated, and plated onto poly-D-lysine and laminin coated 6 cm dishes at 1.5×10^6 cells/dish in Neurobasal A medium (Invitrogen), supplemented with 0.5 mM L-glutamine and B27. One half of the culture media was exchanged with fresh media biweekly.

Immunocytochemistry and fluorescent microscopy

Cells were fixed in 4% formaldehyde at 4°C for 20 min, permeabilized in 0.3% Triton-X-100/PBS for 5 min at 4°C, and blocked in 10% goat serum in PBS for 1 hour at 4°C. Cultures were then incubated overnight in primary antibodies in PBS at 4°C, rinsed 3X in PBS, and incubated in secondary antibodies for 1 hour at room temperature. Following 3 washes in PBS, cultures were mounted onto coverslips with Flouromout G (Southern

Biotechnology). Primary antibodies were as follows: mouse anti-CPG2 monoclonal 200A6.1 (1 μg/ml) and rabbit anti-PSD-95 (1:300; Zymed). Secondary antibodies were as follows: goat anti-mouse Alexa Flour555 (1:500; Molecular Probes) and goat anti-rabbit Alexa Flour647 (1:500; Molecular Probes). Flourescent images of neurons were imported into Spot 3.5 (Diagnostic Instruments) with a Diagnostic Instruments Spot2 digital camera mounted on a Nikon Eclipse E600 using a 60X/1.40 Plan Apo oil immersion objective (Nikon). Images were saved as gray-scale TIFFs and overlayed and pseudo-colored for figures with Photoshop5.0. Scion Image Beta 4.0.2 was used for spine morphology quantifications. Student's T-test was used to compare groups.

RNA interference

5 separate DNA oligonucleotides containing a 19 nucleotide sequence specific to *cpg2*, a 9 nucleotide loop region (TTCAAGAGA), and the 19 nucleotide antisense *cpg2* sequence were annealed to their antisense counterparts and ligated into the pSilencer1.0-U6 plasmid (Ambion) downstream of the U6 promoter. These shRNA constructs were cotransfected with a *cpg2*-IRES-GFP construct into 293T cells at a 40:1 ratio with Lipofectamine2000 (Invitrogen). After 48 hours, cells were fixed and mounted, and GFP fluorescence was examined. One shRNA construct (*cpg2-shRNA*) resulted in >80% reduction in GFP fluorescence intensity (data not shown). The effective RNAi target sequence was GCAGACTGCTGAGATGTAC. For a negative control, a mutated shRNA (*Mcpg2-shRNA*) was generated with the orientation of six interior nucleotides of the target sequence reversed (marked in bold): GCAGAC**AGTCGT**GATGTAC.

Lentivirus generation

For *cpg2* overexpression, the *cpg2* coding region was subcloned into EGFP-C1 (Clontech) downstream of the EGFP sequence. The entire GFP-*cpg2* fusion sequence was subcloned downstream of the ubiquitin-C promoter of the pFUGW lentivirus transfer vector (Lois et al., 2002). For RNAi, the shRNA sequences with the U6 promoter were amplified from the pSilencer1.0 plasmid by PCR and subcloned into the PacI site of pFUGW immediately upstream of the ubiquitin-C promoter, which is upstream of GFP. The clathrin-GFP fusion sequence from EGFP-C1 (Blanpied et al., 2002) was also subcloned into the pFUGW vector downstream of the ubiquitin-C promoter. Lentivirus particles containing these vectors were produced by cotransfecting the transfer vector with the HIV-1 packaging vector Δ8.9 and the VSVG envelope glycoprotein into 293T cells with Lipofectamine2000 (Invitrogen) 24 hours after plating at $15X10^6$ cells/15 cm culture dish. 48-60 hours after transfection culture media was collected and centrifuged at 5000 RPM for 5 min. Media was filtered through 0.4 μm filters (Nalgene) and centrifuged at 25000 RPM for 90 min at 4°C. Supernatants were discarded, and the virus pellets were incubated in 100 μl PBS at 4°C. After 16 hours, viral particles were resuspended, aliquoted, and frozen at -80°C. Viral titers were determined by application of serial dilutions to 293T cells and quantifications of GPF expression 72 hours later. Typical titers were ~$1X10^6$ pfu/μl. For high-density cultures, neurons were infected with viral particles equal to two times the number of plated neurons. For medium-density cultures, particles equal to one half the number of plated neurons were used.

Western blot

For culture Western blotting, high density cortical cultures (1.5 X 10^6 cells/6 cm dish) were infected at 6 DIV with ~3 X 10^6 pfu of *Mcpg2-shRNA* or *cpg2-shRNA* lentivirus. At 21 DIV, cells were lysed with 500 µl of 0.5% SDS-RIPA buffer (1% Triton X-100, 0.5% SDS, 0.5% deoxycholic acid, 50 mM $NaPO_4$, 150 mM NaCl, 2 mM EDTA, 50 mM NaF). Cell lysates were scraped into Eppendorf tubes and centrifuged. Protein was run on an 8% SDS-PAGE gel and probed with the anti-CPG2 monoclonal antibody (3 µg/ml) and a rabbit anti-β-tubulin antibody (1:200; Santa Cruz). 293T cell protein extracts were generated similarly using NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, and 0.5% NP-40). Western blots were probed with mouse anti-Flag (1:1000; Sigma) and rabbit anti-GFP (1:200; Clontech) antibodies.

Immunoelectron microscopy

Hippocampal cultures were processed for NR1 immunostaining for electron microscopic analysis. Cultures were immersion fixed with 4% paraformaldehyde and 0.1% glutaraldehyde. Cultures were then immunostained for NR1 (1:1000; Chemicon) overnight at room temperature. After several washes in phosphate buffer (PB), section were incubated with biotinylated horse anti-mouse secondary antibodies (1:250; Vector) for 2 hours followed by incubation in Avidin biotin-peroxidase (1:200; Vector) for 2 hours at room temperature. Tissue-bound peroxidase was then visualized with a diaminobenzidine (DAB; 5 mg/ml, H_20_2 0.03%) reaction. Coverslips were osmicated, dehydrated and embedded in durcupan (FLUKA, ACM). Ultrathin sections (60 nm thick) were prepared on a Ultracut UCT, Leica ultramicrotome and analyzed in a Tecnai 10 electron microscope. For clathrin-coated pit and vesicle quantifications, neurons randomly selected from unstained *Mcpg2-shRNA* (n = 35 cells) and *cpg2-shRNA* (n = 28

cells) infected cultures were photographed at magnification of 55,400. The number of endocytic pits and vesicles in association with asymmetrical synaptic membrane specializations were quantified blind to the treatment group. Part of the perykarion and adjacent neuropil of cells were analyzed in a 49 μm^2 region/per cell. An endocytic pit or vesicle was noted if it fell within 500 nm distance from an asymmetrical membrane specialization.

Internalization assay

High density cultures were infected at 6 DIV with the *Mcpg2-shRNA* or *cpg2-shRNA* lentivirus vectors. At 21 DIV, cultures were incubated in the protease inhibitor leupeptin (100 μg/ml; Sigma) for 30 minutes. Cultures were then washed in cold DPBS and incubated in 1.5 mg/ml sulfo-NHS-SS-biotin in DPBS for 20 min at 4°C. Unbound biotin was quenched and removed by DPBS plus 50 mM glycine. Cultures were then either left in DPBS at 4°C or returned to their original media (plus leupeptin) and incubated at 37°C for 30 min. Extracellular biotin was then cleaved with 50 mM glutathione, 90 mM NaCl, 10 mM EDTA, 75 mM NaOH, 1% BSA, pH 8.75, which was quenched by DPBS plus 5 mg/ml iodacetamide. Calibration cultures were kept at 4°C in DPBS, without stripping. Cultures were then lysed in 0.5% SDS-RIPA buffer and scraped into eppendorf tubes, and protein extracts were centrifuged. Protein concentrations were determined with BCA (Pierce). 300 μg of protein was added to 1 ml 0.1% SDS-RIPA buffer, and biotinylated proteins were isolated with neutravidin-agarose slurry (Pierce). Neutravidin-agarose beads were washed in 0.1% SDS-RIPA buffer and boiled in SDS-PAGE sample buffer. Pelleted proteins were run on an 8% SDS-PAGE gel, transferred to nitrocellulose, and probed with monoclonal antibodies against NR1 (1:500; Chemicon), GluR2 (1:1500;

Chemicon), and insulin receptor, β-subunit (1:200; Oncogene). For the total protein control, 30 μg of protein from each unpelleted sample was probed for NR1, GluR2, and CPG2. For total surface biotinylation assay, 75 μg of unstripped surface biotinylated proteins from *Mcpg2-shRNA* and *cpg2-shRNA* infected cultures were pelleted with neutravidin, run on an SDS-PAGE gel next to 30 μg of total protein, and probed for NR1 and GluR2. Western blot images were quantified with Scion Image Beta 4.0.2, and student's T-test was used to compare groups.

Figure 4-1. *cpg2-shRNA* knocks-down exongenous CPG2 in 293T cells

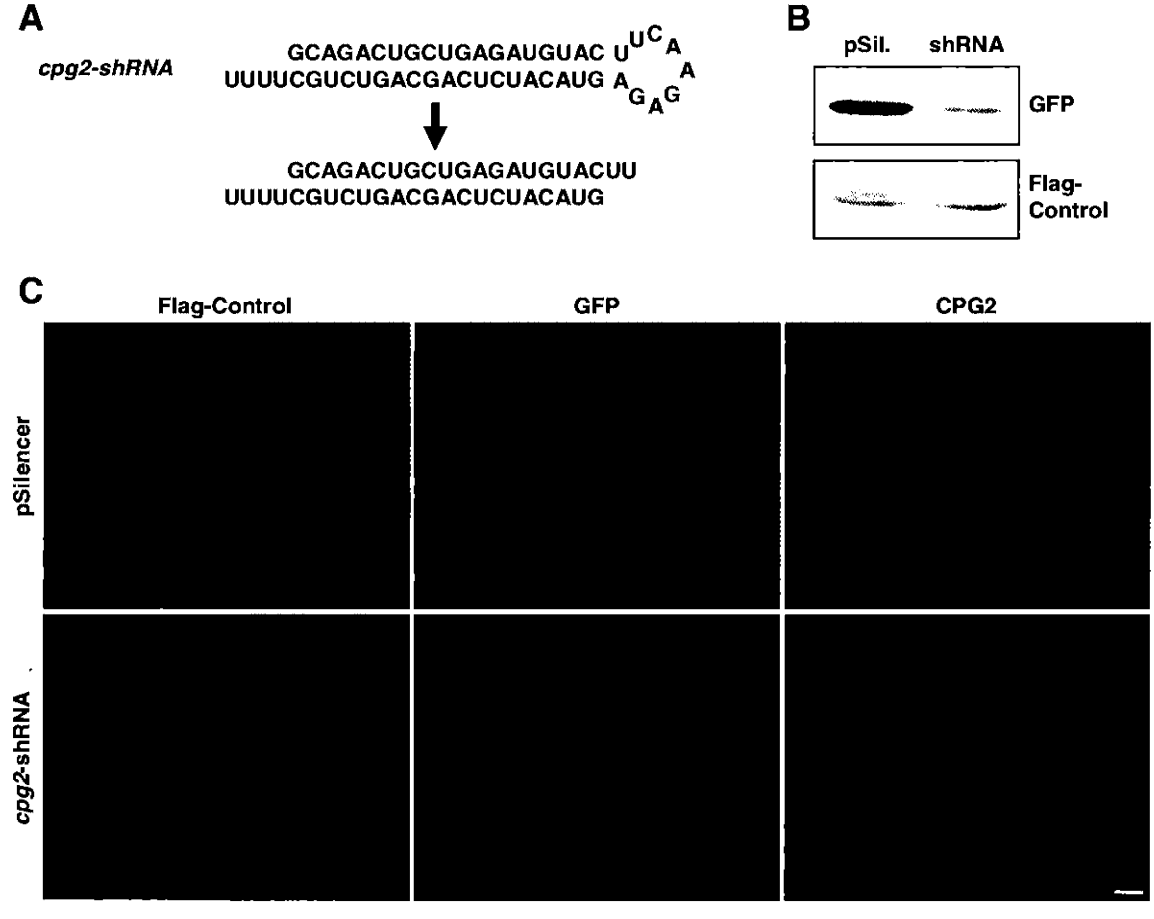

Figure 4-2. *cpg2-shRNA* knocks-down endogenous CPG2 in neurons

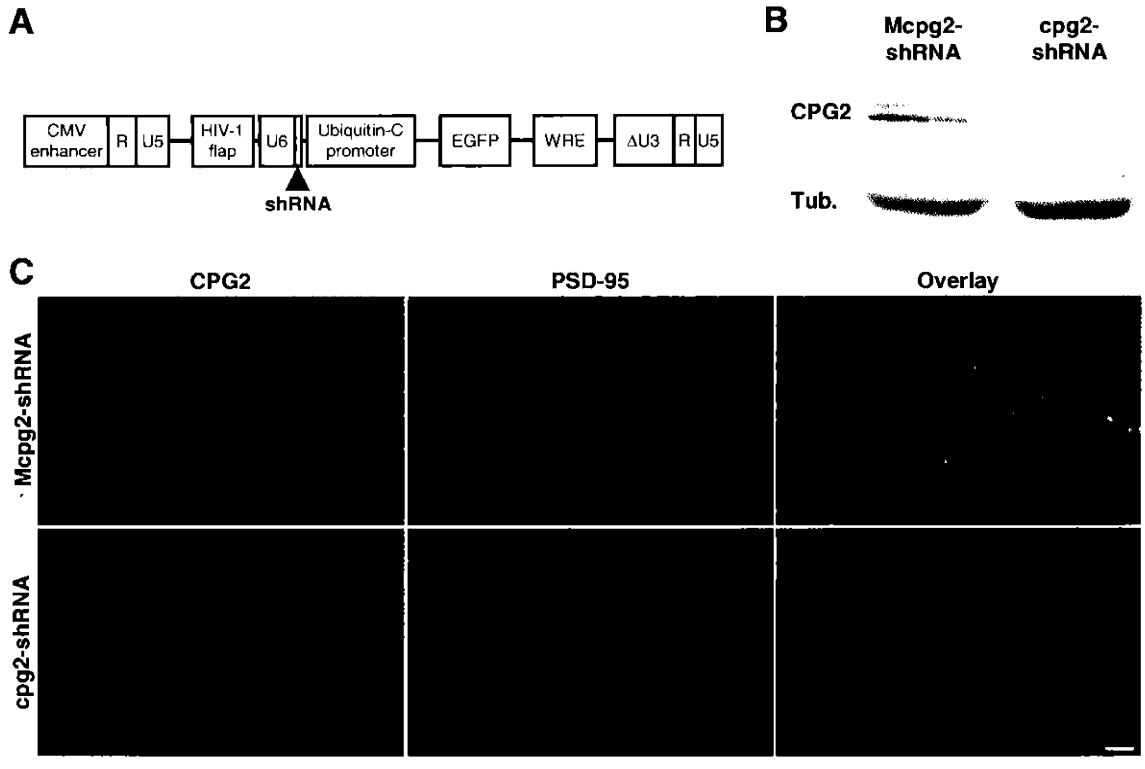

Figure 4-3. Effects of CPG2 knock-down and overexpression on spine morphology

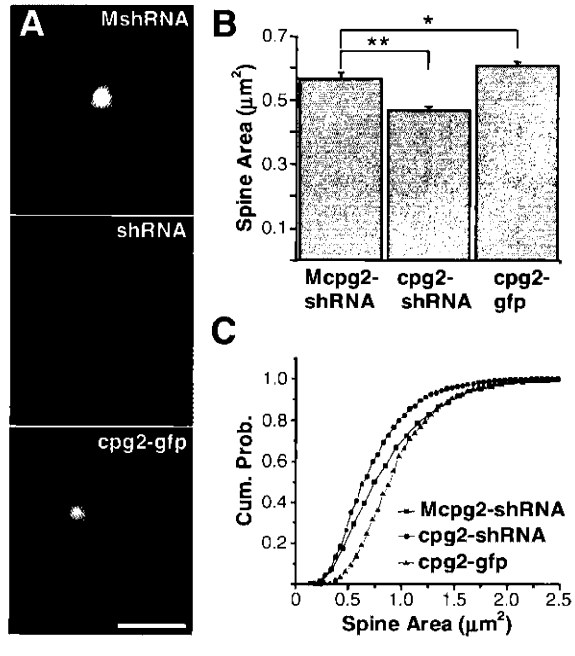

Figure 4-4. CPG2 knock-down increases number of postsynaptic clathrin-coated pits and vesicles

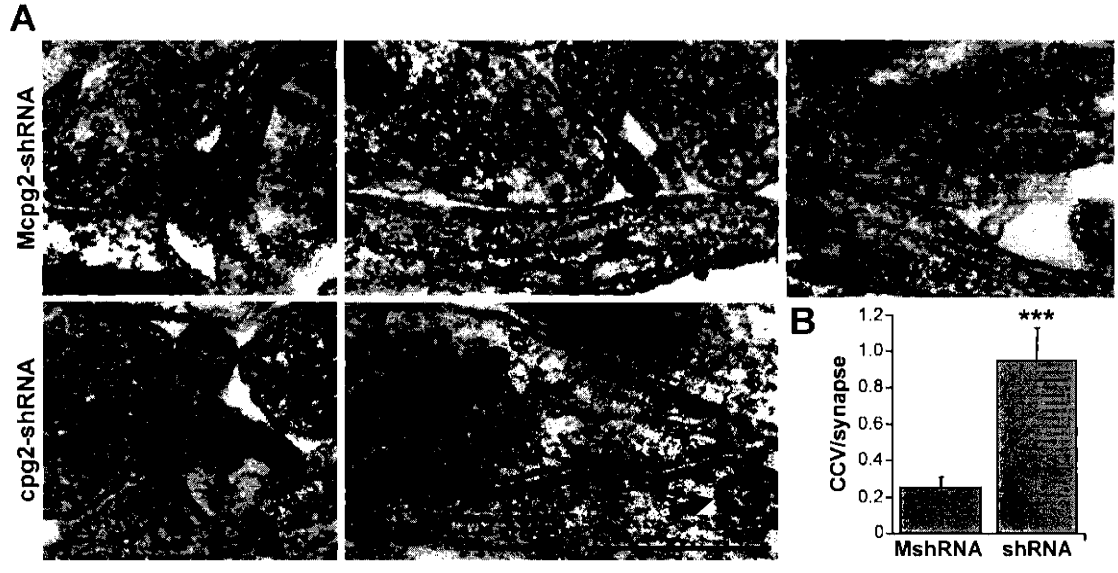

Figure 4-5. CPG2 knock-down increases the number of NMDA receptor trafficking vesicles

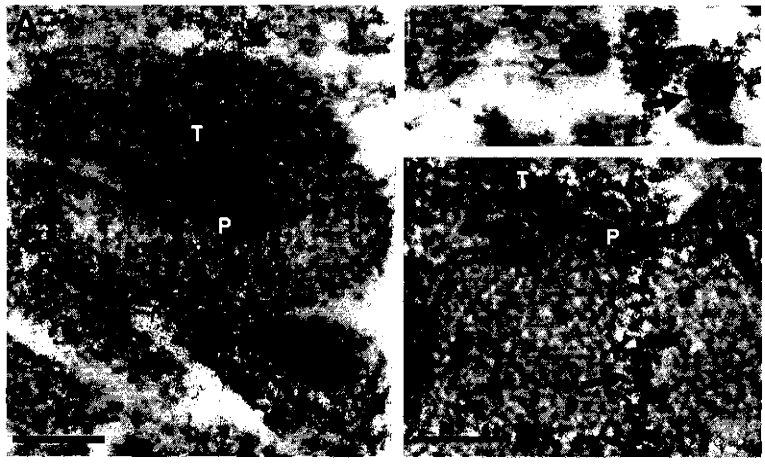

Figure 4-6. CPG2 knock-down disrupts glutamate receptor internalization

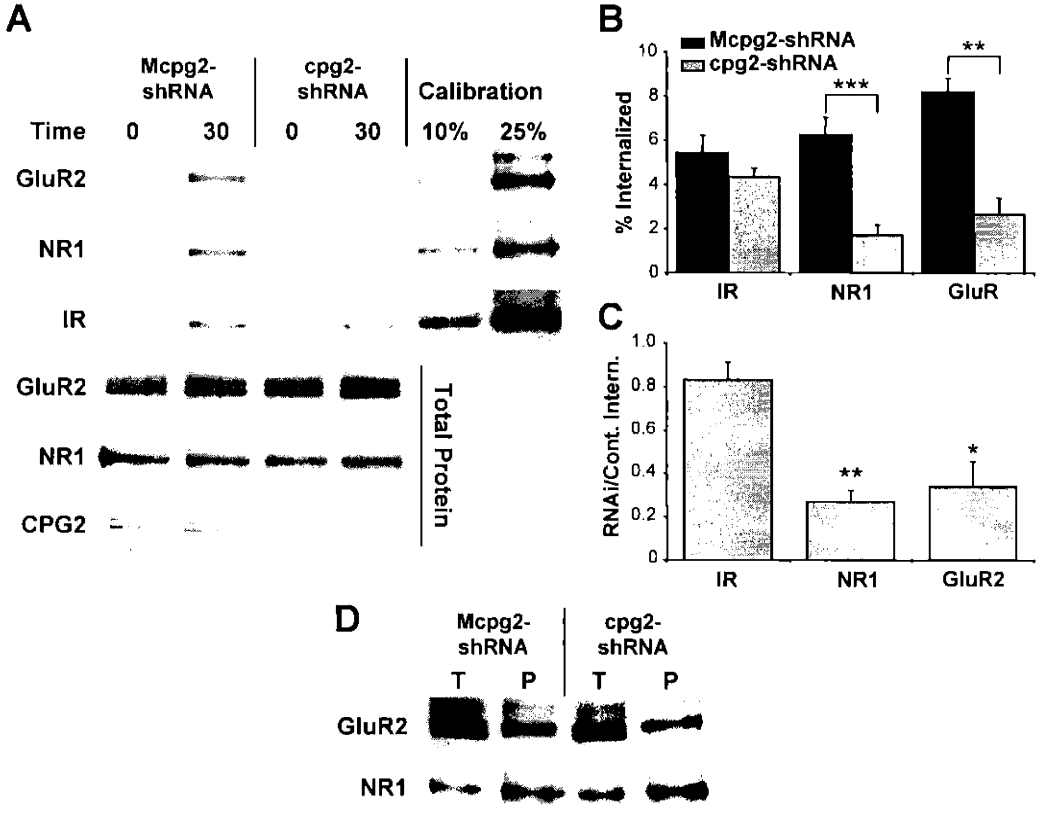

Figure 4-1. *cpg2-shRNA* knocks-down exongenous CPG2 in 293T cells

(A) Schematic of *cpg2-shRNA* expressed in 293T cells from the pSilencer1.0 plasmid and the resulting siRNA. (B) Western blot of protein extracts from 293T cells transfected with *cpg2-IRES-GFP*, a plasmid expressing a Flag-tagged control protein, and either (left) pSilencer alone or (right) *cpg2-shRNA* in pSilencer, probed with antibodies against GFP and Flag. (C) 293T cells were transfected with *cpg2-IRES-GFP*, a Flag-tagged control plasmid, and either pSilencer or *cpg2-shRNA* fixed and stained for Flag and CPG2. Scale bar: 20 μm.

Figure 4-2. *cpg2-shRNA* knocks-down endogenous CPG2 in neurons

(A) Schematic of *cpg2-shRNA*-pFUGW lentivirus transfer vector construct. (B) Western blot of proteins from cortical cultures infected with *Mcpg2-shRNA* or *cpg2-shRNA* probed for CPG2 and tubulin. (C) Cultured hippocampal neurons were infected with *Mcpg2-shRNA* (top) or *cpg2-shRNA* (bottom) at 4DIV and fixed and stained at 24DIV for CPG2 and PSD-95. Scale bar: 5 μm.

Figure 4-3. Effects of CPG2 knock-down and overexpression on spine morphology

(A) Individual dendritic spines from neurons infected with *Mcpg2-shRNA* (MshRNA), *cpg2-shRNA* (shRNA), or *cpg2-gfp.* Spine areas from each group were measured and plotted by their mean plus SEM (B) and cumulative probability (C).

Figure 4-4. CPG2 knock-down increases the number of postsynaptic clathrin-coated pits and vesicles

(A) EM micrographs of synapses from *Mcpg2-shRNA* (top) and *cpg2-shRNA* (bottom) infected neurons on dendritic spines (left) and shafts (middle, right). Arrows = coated vesicles; S = spine; T = presynaptic terminal; Scale bar: 500 nm. (B) Quantification of the number of synapse-associated clathrin-coated pits and vesicles (CCV) per synapse in *Mcpg2-shRNA* and *cpg2-shRNA* infected neurons.

Figure 4-5. CPG2 knock-down increases the number of NMDA receptor trafficking vesicles

(A,B) Preembedding immunostaining for NR1 on *cpg2-shRNA* infected neurons shows that the presence of NMDA receptors in synapse-associated clathrin-coated vesicles (arrow). Arrowhead shows unstained vesicle. (C) Unstained negative control.

Figure 4-6. CPG2 knock-down disrupts glutamate receptor internalization

(A) Reversible biotinylation glutatmate receptor internalization assay. Surface proteins were biotinylated, cultures returned to normal media at 37°C or kept at 4°C, and remaining surface biotinylation was stripped. Biotinylated proteins were isolated and probed for GluR2, NR1, and insulin receptor, subunit β. Bottom: To check for changes in total protein levels, total protein was loaded and probed for GluR2, NR1, and CPG2. (B,C) Quantification of internalization assay showing the effects of CPG2 knock-down on receptor internalization (B) and its percent inhibition of receptor internalization relative to the control neurons (C). (D) Surface protein from *Mcpg2-shRNA* and *cpg2-shRNA* infected neurons were biotinylated and isolated with neutravidin. Biotinylated proteins were run next to total proteins on an SDS-PAGE gel and probed for GluR2 and NR1.

Chapter 5

Conclusions

The formation of long-term memories and long-term potentiation (LTP) require both the transcription of new mRNAs and the translation of new proteins (Flexner et al., 1963; Frey et al., 1993; Goelet et al., 1986; Huang and Kandel, 1994; Huang et al., 1994; Nguyen et al., 1994). The immediate early genes (IEGs) are the primary genomic response to activity that triggers the synaptic rearrangements underlying synaptic plasticity (Nedivi, 1999; Sheng and Greenberg, 1990). Many encode transcription factors that likely stimulate the transcription of downstream genes that effect synaptic plasticity. *cpg2* was first isolated in a screen for these plasticity-effector genes (Nedivi et al., 1993), and its expression pattern is suggestive of a role in neuronal function and synaptic plasticity (Nedivi et al., 1996). In this thesis, I described my studies to understand the regulation and function of *cpg2* in neurons. I showed that *cpg2* is a splice-variant of the *syne-1* gene whose expression is restricted to regions of the brain capable of robust synaptic plasticity, suggesting that it plays a unique role in the neurons of these brain regions. The CPG2 protein is localized specifically to the postsynaptic endocytic zone of excitatory synapses, often in the vicinity of clathrin-coated pits. At this endocytic zone, CPG2 underlies the organization of a network of proteins including the actin cytoskeleton and endophilin B2, a member of a family of membrane-trafficking proteins. Lastly, using

RNAi to knock-down its levels, I showed that CPG2 is an important regulator of the internalization of glutamate receptors.

Glutamate receptor internalization is critical for the maintenance and modification of synaptic strength (Carroll et al., 2001; Malinow and Malenka, 2002) and likely occurs through clathrin-mediated endocytosis (Beattie et al., 2000; Carroll et al., 1999; Ehlers, 2000; Lin et al., 2000; Man et al., 2000). The basic machinery of clathrin-mediated endocytosis is conserved across species and cell types (Schmid, 1997). However, in cases where specialized endocytic functions are required, specific mechanisms have evolved for regulating endocytosis. In the presynaptic terminal, for example, there are novel mechanisms and proteins for meeting the demands of synaptic transmission, often consisting of terminal-specific isoforms or splice-variants of general endocytic proteins (Jarousse and Kelly, 2001; Slepnev and De Camilli, 2000). Given its importance for synaptic function and plasticity, neurons may have developed novel mechanisms for regulating the endocytosis of glutamate receptors. A postsynaptic endocytic zone has been shown to be present and stable on dendritic spine and shaft synapses (Blanpied et al., 2002). Although segregated from the PSD, it is likely an integral synaptic component (Blanpied et al., 2002), and data presented here strongly suggest that it is the site of glutamate receptor internalization. Thus far, all proteins shown to be necessary for glutamate receptor internalization, including actin (Zhou et al., 2001), dynamin (Carroll et al., 1999; Luscher et al., 1999), and huntingtin interacting protein 1 (Metzler et al., 2003), are proteins in the general endocytic pathway (Slepnev and De Camilli, 2000). CPG2 is the first protein known to be unique to the postsynaptic endocytic zone and the first identified specific regulator of the endocytosis of postsynaptic proteins, including

glutamate receptors. I hypothesize that CPG2 is a component of a specialized endocytic mechanism dedicated to the internalization of postsynaptic proteins. Since CPG2 knock-down did not affect the total number of surface glutamate receptors, neurons do not require it for normal expression of synaptic proteins. As the product of an activity-regulated transcript expressed solely in brain regions with significant synaptic plasticity mechanisms, CPG2 may underlie a novel adaptation of the clathrin-mediated endocytosis pathway that enables the capacity for postsynaptic plasticity in these synapses.

The phenotype in CPG2 knock-down neurons is similar to that observed in the presynaptic terminals of *Drosophila* with endophilin mutations. At these sites, there is an accumulation of endocytosed clathrin-coated synaptic vesicles and a disruption of neurotransmitter release, due to the inability of the vesicles to reenter the vesicle cycle (Fabian-Fine et al., 2003; Verstreken et al., 2002; Verstreken et al., 2003). This phenotype results from the fact that endophilin recruits synaptojanin, an enzyme that catalyzes vesicle uncoating, to endocytosed synaptic vesicles (Brodin et al., 2000; Verstreken et al., 2003). Synaptojanin I mutant mice show an identical deficit in the synaptic vesicle cycle, suggesting a similar role at the mammalian presynaptic terminal (Cremona et al., 1999; Kim et al., 2002). Based upon these data, I propose a model for the function of CPG2 in the endocytosis of glutamate receptors (Figure 5-1, left). CPG2 is localized to the endocytic zone of excitatory synapses, where it organizes a protein network, including endophilin B2 and the actin cytoskeleton via the coiled-coils in its C-terminus. For internalization to occur, glutamate receptors are dissociated from their anchorage in the PSD and translocated to the endocytic zone, where they are internalized by clathrin-mediated endocytosis. Once internalized, CPG2 promotes the downstream

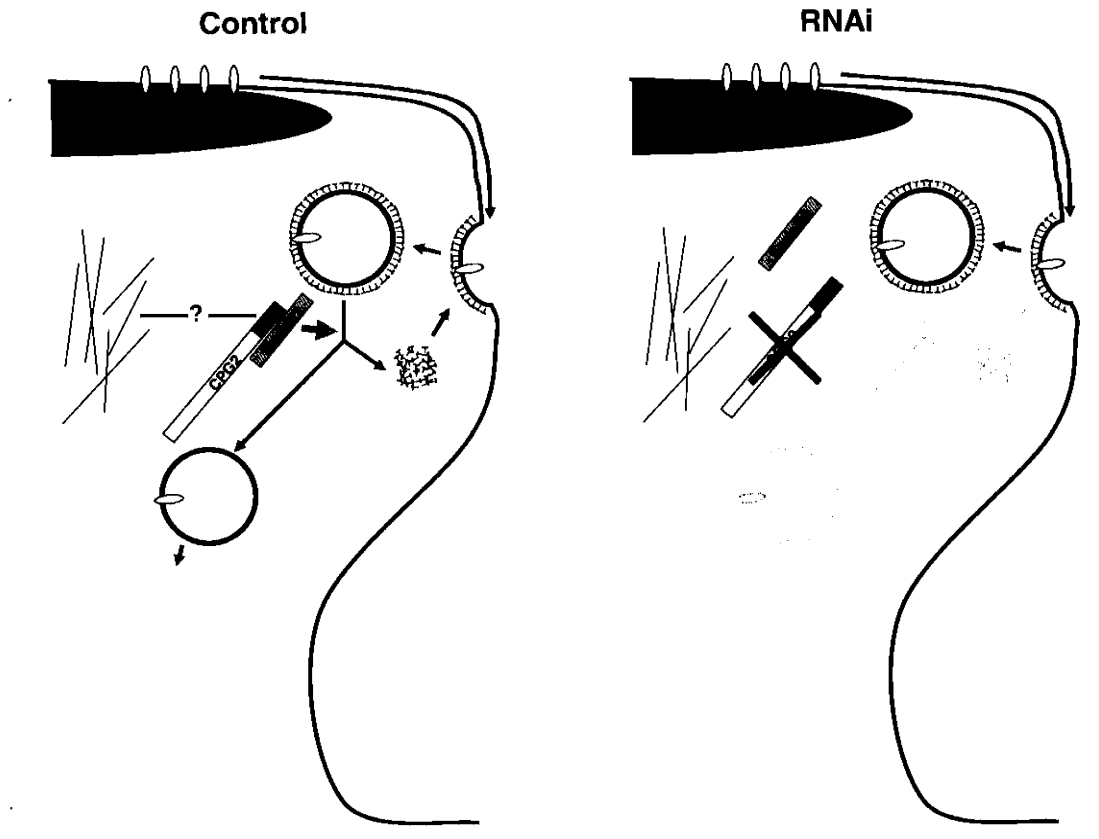

Figure 5-1. Model of CPG2 function

(Left) At excitatory synapses, glutamate receptors are translocated (red arrow) to the endocytic zone. Receptors are internalized via clathrin-coated vesicles. Vesicles are uncoated and the endocytic proteins are returned to the endocytic zone for further receptor internalization. CPG2 forms a protein complex with the actin cytoskeleton (black lines) and endophilin B2 at the endocytic zone, where it likely regulates a late step of the vesicle trafficking pathway, such as uncoating. (Right) In CPG2-knock-down neurons, clathrin-coated vesicles accumulate and glutamate receptor internalization is disrupted, by the removal of the CPG2 protein network.

processing, such as the uncoating, of the vesicle through its interaction with endophilin B2 or other endocytic proteins, allowing the vesicle to be trafficked to the endosome and the clathrin-coat components to return to the endocytic zone to repeat the process. Following RNAi-mediated CPG2 knock-down, the CPG2-associated protein network is disrupted, inhibiting the processing of postsynaptic clathrin-coated vesicles, and, therefore, causing the accumulation of these vesicles (Figure 5-1, right). Due to the

slowed kinetics of this reaction, the entire endocytic pathway is slowed, disrupting glutamate receptor internalization.

cpg2 was isolated in a screen for seizure-induced genes and is regulated by physiological activity (Nedivi et al., 1996; Nedivi et al., 1993). In general, screens for activity-regulated genes have isolated a number of synaptic proteins that are the components of the basic transmission machinery, indicating that the synaptic rearrangements that occur during plasticity likely involve an augmentation of normal synaptic processes (Nedivi, 1999). Since CPG2 is important for normal synaptic function, an increase in *cpg2* expression may belie an increase in synapse formation during synaptic plasticity. However, following LTP, there is an increase in the number of clathrin-coated pits and vesicles in dendritic spines, which may reflect an enhancement of postsynaptic protein cycling during plasticity (Toni et al., 2001). A role for CPG2 in postsynaptic endocytosis may explain the increased need for *cpg2* expression during periods of synaptic plasticity.

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