ROLE OF CYCLIC GMP-DEPENDENT PROTEIN KINASE TYPE II IN THE BRAIN

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Neuroscience,

UBC THE UNIVERSITY OF BRITISH COLUMBIA

November 2004

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ABSTRACT

PKG is one of the most important downstream effector of the NO/natriuretic peptides cGMP pathway. Of the two types of PKG, within the brain PKG I is almost exclusively expressed in the cerebellum. By contrast PKG II has a much wider expression in the brain, indicating that it is most likely to be the mediator of cGMP actions. However, subcellular distribution and the function of PKG II in the brain is still unknown.

Western blot analysis using a PKG II specific antibody confirmed previous reports that PKG II is highly expressed in the cortex, striatum, hippocampus, hypothalamus and thalamus and absent in the cerebellum. In comparison, we found that PKG I is highly expressed in the cerebellum and hypothalamus and very weakly expressed in the hippocampus and cortex.

Western blot analysis of subcellular fractions from adult brain shows that PKG II is present in both the pre and postsynaptic fractions and is enriched in the membrane fractions. PKG II is also enriched in the synaptosomal membrane fraction of embryonic rat brain subcellular fractions. Immunocytochemisty using the PKG II antibody indicates that PKG II is present in both the cell body and processes of cortical, hippocampal and thalamic neurons in culture. PKG II was found to co-localize with both the presynaptic marker, synaptophysin and the postsynaptic marker PSD-95, indicating that PKG II is present at the synapse.

Using wild type (wt) and non-myristoylated mutant PKG II proteins it was demonstrated that N-myristoylation (N-myr) is important for targeting of PKG II to membranes and distal ends of filopodia like structures in COS-1 and HEK-293 cells. Full length wt PKG II and wt

regulatory PKG II proteins, when overexpressed in hippocampal neurons targeted to the synapse, however, a G2A PKG II mutant, which does not undergo myristoylation had a much more diffuse distribution in the entire cell and did not target to the synapse. This indicates that N-myr is important for proper targeting of PKG II.

Overexpression of the dominant negative regulatory domain of PKG II in hippocampal neurons caused a 2.5-fold increase in filopodia in young neurons and a 2-fold increase in spine like structures in older neurons compared to GFP overexpressing cells. Such an effect was not observed with the FLwt PKG II. The non-myristoylated forms of PKG II overexpressing cells did not show an increase in filopodia/spine like structures, indicating that proper targeting of PKG II through myristoylation is important for the regulatory domain to have a dominant negative effect. This indicates that PKG II is most likely to play a role in neuronal development and regulation of synaptogenesis.

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List of Abbreviations

Ab	antibody
bp	base pair
BSA	bovine serum albumin
C	Celsius
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	adenosine 3', 5'-cyclic monophosphate
cat	catalytic
Cat. No.	Catalogue Number
cDNA	complementary DNA
cGMP	guanosine 3', 5'-cyclic monophosphate
DMEM	Dulbeccos's-modified Eagle medium
DMSO	di-methyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethylether) N,N,N',N' tetraacetic acid
FBS	foetal bovine serum
FL	full length
g	gram
GFP	green fluorescent protein
HBSS	Hank's balanced salt solution
HEK-293	human embryonic kidney-293 cells
h	hour/s
IP	immunoprecipitation
kb	kilo base
KCl	potassium chloride
kDa	kilo Daltons
KLH	keyhole limpet haemocyanin

L	litre
LTD	long-term depression
LTP	long-term potentiation
M	molar
mA	milli amperes
$MgCl_2$	magnesium chloride
min	minute
mL	milli litre
mM	milli Molar
mm	milli meter
MMLV	murine mouse leukaemia virus
MW	molecular weight
mRNA	messenger RNA
mut	mutant
Na	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
NBM	Neurobasal medium
NGS	normal goat serum
ng	nano gram
nm	nano metre
nM	nano Molar
NM	non-myristoylated
NO	nitric oxide
NOS	nitric oxide synthase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C

PKG I	cyclic GMP-dependent protein kinase type I
PKG II	cyclic GMP-dependent protein kinase type II
pM	pico Molar
PMSF	phenyl methyl sulfonyl fluoride
RFP	red fluorescent protein
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcription
SDS	sodium dodecyl sulphate
sec	seconds
snt	supernatant
Taq	thermus aquaticus
TEEN	tris-EDTA-EGTA-NaCl
temp.	temperature
Tris	tris (hydroxymethyl) aminomethane
U	Units
UBC	University of British Columbia
μL	micro litre
μg	micro gram
μM	micro Molar
V	volts
vol	volume
wt	wild type

Dedication

This thesis is dedicated to all those animals that unwillingly laid down their lives to add another drop of information to the vast biological sciences ocean.

Acknowledgement

There are numerous people I owe thanks to and I would like to name them in the order in which they entered my science life.

The reason I am in biological sciences is probably because of the influence of the works of Gerald Durrell and Richard Dawkins. I have great admiration for their work and their apparent love for science. Their talent has definitely made science so much more beautiful than it already is.

I extremely thank my parents and my brother for their moral and financial support, without which it would have been impossible for me to come to Canada to pursue higher education. I would like to thank them for their understanding, for having faith in me and for encouraging me in spite of my choosing a career that they did not quite approve of.

I would like to thank Dr. Tim Murphy for allowing me to work in his lab when I first came to Vancouver and helping me taste my first research experience in Canada. I am also grateful to Tim for his advice and numerous insightful discussions while I was taking the Neuroscience courses and during lab meetings.

I am indebted to Dr. Paul Mackenzie, a former student of Dr. Tim Murphy, for helping me get used to the ways of Kinsmen lab and life in Canada in general. I would have been totally lost if it hadn't been for Paul's advice and help in finding a supervisor for graduate studies.

The primary reason for my having gotten this far is because of Dr. Steven R. Vincent, my supervisor. To be honest I have no idea what was going through his mind when he accepted me as a graduate student as I

had no prior practical experience in molecular biology. Steve has been tremendously patient in spite of my occasionally trying his patience beyond endurance and has shown an unbelievable amount of faith in me for I can't even remember how long now. His unconditional support, guidance, scientific or otherwise and encouragement throughout my stay in his lab would be the primary reasons for my getting a Ph.D. degree. I am most grateful to Steve for letting me carry out my research in an independent manner. I cannot thank Steve enough for everything he has done for me and even if I could, I believe English language is deficient enough of an expression to communicate my complete gratitude.

I would like to express my gratitude to Liz Wong for the great help in dealing with course registration, paper work and other seemingly simple tasks pertaining graduate studies, which actually required a lot of time and effort.

One of the greatest pleasures I have had in Kinsmen lab is the company of Dr. Alaa el-din El-husseini, initially as a graduate student in Steve's Lab and for the last three years as a faculty member in Kinsmen Lab. Although I believe that a graduate student has to motivate himself/herself from within, it is impossible to miss someone like Alaa, who I find to be an infinite source of motivation to do science. I extremely thank Alaa for teaching me techniques in molecular biology when I first joined the lab, for numerous intellectual discussions, for encouragement and motivation. I also would like to thank him for his wonderful friendship and all the delightful science unrelated conversations we had, which were a result of our mutual admiration for the opposite sex.

It was an immense privilege to work in the same department as Dr. Lynn A. Raymond. I have always tried to incorporate the indefatigable nature of Lynn in my work, but I believe I still have a long way to go. I am indebted to Lynn for intuitive discussions, advice during the Neuroscience courses, during lab

meetings and the supervisory committee meetings. I am eternally grateful for the support and encouragement that Lynn provided me during my degree, especially during my comprehensive examination.

I would like to thank Dr. Peter Reiner and my supervisory committee members Dr. Steven Pelech and Dr. Timothy O Connor for their enormously useful advice, valuable suggestions and encouragement.

I would like to express my gratitude to Pascale Fretier for her friendship and technical assistance, which was invaluable. It was a pleasure to have the company of Pascale with whom I could share my adventures outside the laboratory over a coffee.

I would like to thank Cindy for her technical assistance and the much required smiling face to perk you up during depressing rainy days in Vancouver.

I would like to acknowledge the exceptional friendship of Dr. Oliver Prange, Dr. Heather Guthrie, Brett Abrahams, Catriona Wilson, Chu-see, Dr. Bruce Connop, Dr. Stefan le behan, Dr. Dorota Kwasnicka, Dr. Melinda Zeron, Tim Blanche, Dr. Tara Stewart, Shaheen Mohammadi, Ruzbeh Shooshtarian, Andy Laycock, Dr. Helen Fleisig, Andy Shih, Zhi Liu, Catherine Gauthier-Campbell, Josh Levinson, Christina Cheng, Jackie Shehadeh, Herman Fernandez, Alicia Davis, Kimberly Gerrow, Sophie Imbeault, Pam Aristakaitis, Dr. Joseph Pathakamuri and the darling of our lab David Lin for enriching the Kinsmen laboratory environment and making it an absolutely fabulous place to work at.

I would also like to thank the UBC Cricket Club members for giving me an opportunity to play cricket, a game that I absolutely adore and enjoy, and for their friendship, which helped keep my body sound to go with my rarely sound mind. Most important I owe my thanks to them for opening the doors to the world

Introduction

I. NO introduction required

NO (molecular mass 30 Da) is one of the ten smallest molecules found on earth (Lincoln J et al., 1997). Since the discovery in the 1980s, that the free radical NO could be produced by mammalian cells and can act both as a physiological messenger and a pathophysiological agent, it has opened a whole new field of biological research (Moncada et al., 1991). It was in 1977 that NO was identified as the therapeutically effective compound released from drugs that had been, for over one hundred years, used in medicine to treat cardiovascular diseases (Katsuki et al., 1977). A few years later Furschgott and Zawadzki discovered the presence of a smooth muscle relaxing factor, which they named Endothelial derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Seven years later EDRF was shown to have similar properties to NO (Ignarro et al., 1987; Palmer et al., 1987). NO is synthesized from the amino acid L-Arginine by the family of enzymes known as NO synthases (NOS). Three different isoforms of NOS have been cloned so far, the endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) (Bredt et al., 1991; Marsden et al., 1992; Michel and Lamas, 1992; Nishida et al., 1992; Sessa et al., 1992; Xie et al., 1992). NOS uses three co-substrates, arginine, molecular O_2, and NADPH, and converts arginine to NO and citrulline (Dawson and Snyder, 1994; Knowles et al., 1989; Nathan, 1992). In the absence of arginine NOS can convert soluble nitroblue tetrazolium salt to an insoluble, visible formazan in an NADPH-dependent manner (Hope et al., 1991; Schmidt et al., 1993). The NADPH diaphorase histochemistry was commonly used to detect NO producing neurons in the brain.

Since its discovery, NO has been shown to be involved in numerous physiological processes such as smooth muscle relaxation, neurotransmission, platelet aggregation and host defense mechanisms [for reviews see (Krumenacker et al., 2004; Nathan, 1992; Snyder and Bredt, 1992; Vincent, 1994).

II. NO targets

NO brings about its effect in many ways including nitrosylation, ADP-ribosylation of proteins, and mainly by stimulation of cGMP synthesis via soluble guanylyl cylcases (sGC) (Schlossmann et al., 2003). NO targets are mainly metal- and thiol- containing proteins and low molecular weight thiols [for reviews see (Anggard, 1994; Stamler, 1994)]. NO can interact with thiols to form nitrosothiols. Nitrosylation of thiols or thiol containing proteins has been suggested to be a mechanism by which NO can be transported in a stable form, thereby extending its range as a physiological messenger (Anggard, 1994; Stamler, 1994). Axonal proteins GAP-43 and SNAP-25, which are involved in axon growth and synaptogenesis, have been shown to interact with NO and undergo nitrosylation. The nitrosylation of these proteins results in reduced fatty acylation of these proteins. It is thought that reactions of this type could play a role in axonal remodelling during development to establish specific neuronal connections (Hess et al., 1993). Protein nitrosylation has also been shown to be important in regulation of transcription factors like *c-Jun,* NF-κB and the oncogene p21Ras (Klatt et al., 1999; Lander et al., 1995; Matthews et al., 1996). Nitrosylation is thought to be an important regulatory event and recently a highly conserved de-nitrosylating enzyme has been described, indicating that it is likely to be a

reversible process (Liu et al., 2001). High levels of NO have been shown to cause nitrosylation of the glycolytic enzyme, glyceraldhehyde-3-phosphate dehydrogenase (GADPH), which leads to ADP-ribosylation of GADPH. This results in inhibition of the enzyme and it is suggested that NO mediates its cytotoxic effects by this mechanism as it would reduce the capacity of the target cell for energy production (Brune et al., 1994). Thus nitrosylation appears to be important for normal physiological function and also mediates cytotoxicity.

NO combines with O_2 to form nitrite and it is excreted in this form (Anggard, 1994). NO can form peroxynitrite by reacting with superoxides. This ultimately leads to production of hydroxyl radicals (Beckman et al., 1994). In the presence of high levels of NO and superoxides, peroxynitrite and hydroxyl radicals are most likely to form and these are more toxic than NO itself. The hydroxyl radical is a powerful mutagen and peroxynitrite causes extensive protein tyrosine nitration (Beckman et al., 1994; Dinerman et al., 1993; Lipton et al., 1993). Tyrosine nitration has been shown to inhibit the activity of the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA2a) and decrease the activity of prostacyclin synthase (Viner et al., 1999; Zou et al., 1998).Tyrosine nitration appears to be a specific event as Viner et al. showed that SERCA2a is nitrated but not SERCA1 (Viner et al., 1999).

NO exerts most of its physiological effects by activating soluble guanylyl cyclase (sGC) and subsequently elevating cGMP (Knowles et al., 1989; Schmidt et al., 1993). NO is the most potent activator of sGC and low levels of NO are sufficient for enzyme activation (Lincoln J et al., 1997). sGC is expressed in the cytoplasm of almost all mammalian cells. It is a

heterodimer consisting of α and β subunits, both of which are required for catalytic activity. Two isotypes and different subunit compositions have been found for sGC [for review see (Lucas et al., 2000). The NO-cGMP pathway is important for a number of physiological processes such as endothelium dependent relaxation of smooth muscle, inhibition of platelet aggregation and adhesion, neuronal signaling, nitrergic inhibitory transmission in the gastrointestinal, urogenital and cardiovascular systems, pressure-induced natriuresis and tubulo-glomerular feedback in the kidney and modulation of force development in fast-twitch skeletal muscle fibers (Lincoln J et al., 1997).

III. Natriuretic Peptides (NP)

cGMP levels can also be elevated by a family of widely distributed polypeptides called natriuretic peptides [for review see (Baxter, 2004; Kuhn, 2004; Levin et al., 1998)]. The natriuretic peptide family consists of three homologous members, atrial (ANP, B-type (BNP) and C-type (CNP) natriuretic peptides. Guanylin and uroguanylin are other related peptides that can also elevate cGMP. A pro-ANP peptide releases a 98 amino acids and a 28 amino acids fragment which is mature ANP. It circulates in the plasma and is also expressed in the ventricles of fetuses and neonates and in the kidney. BNP, which was originally identified from porcine brain, is present in the plasma. Pro-BNP contains 108 amino acids and releases a mature form which is 32amino acids. Two CNP molecules, 22 and 53 amino acids long, have been identified *in vivo*. The 22 amino acids is predominantly expressed in the central nervous system (CNS), anterior pituitary, kidney, vascular endothelial cells, and plasma. The related peptides guanylin and uroguanylin are 15 and 16 amino acids, respectively, and are

produced in the gastro-interstinal mucosa. The NPs have diverse biological roles including maintenance of normal blood pressure and volume, growth-moderating functions in the heart, renal sodium secretion, and cellular proliferation and differentiation in different tissues (Kuhn, 2004). NPs bring about their effect by activating membrane or particulate GC (pGC). Three pGC activated by NPs have been identified so far, NPR-A (GC-A), NPR-B (GC-B) and NPR-C (GC-C), all of which catalyse the conversion of GTP to cGMP (Garbers, 1991; Garbers and Lowe, 1994). Four other pGC have also been reported, however, they are orphan receptors with no extracellular ligands known so far (Lucas et al., 2000). The major target of cGMP downstream of NPs is PKG, which most likely phosphorylates substrates and mediates NPs actions (Baxter, 2004; Kuhn, 2004; Levin et al., 1998). Recently DiCicco-Bloom et al. showed by *in situ* hybridization the presence of NPs and their receptors NPR-A, NPR-B and NPR-C in embryonic mouse brain and they suggested that the NPs might have an important role in region and stage-specific development of the peripheral and central nervous system (DiCicco-Bloom et al., 2004).

IV. cGMP Targets

cGMP brings about its effects through a) direct channel gating (opening of inward Ca2+ and Na channels); b) cGMP-dependent phosphodiesterases, which leads to regulation of cyclic nucleotide levels; c) ADP ribosyl cyclase and d) PKG, which is the principal intracellular mediator of cGMP signaling (Garthwaite and Boulton, 1995). **The focus of my thesis research was on the NO/NPs-cGMP pathway and its downstream effector PKG in neuronal signaling.**

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V. PKG

1. PKG Genes

PKG is found in various eurkaryotic organisms. PKG activity was initially reported in arthropods (Kuo and Greengard, 1970) and since then it has been identified in *silkworm*, *Paramecium* (Miglietta and Nelson, 1988), *Tetrahymena* (Murofushi, 1974), *Dictyostelium discoideum* (Wanner and Wurster, 1990), *Caenorhabditis elegans* (Stansberry, 2001 #142) and two genes DG1 and DG2 in *Drosophila* (Kalderon and Rubin, 1989). A soluble mammalian form of PKG was first described (Hofmann and Sold, 1972) followed by isolation of a membrane-bound PKG from intestinal epithelium (de Jonge, 1981), which led to the hypothesis that two forms of PKG existed, type I and type II [for review see (Pfeifer et al., 1999)]. PKG I was cloned from bovine (Wernet et al., 1989) and human smooth muscle (Sandberg et al., 1989), which revealed that PKG I exists in two alternatively spliced isoforms 1α and 1β. The two spliced isoforms differ only in their first ~80 aa at the N-terminus (Ruth et al., 1997; Wernet et al., 1989) and the rest of the molecule is identical. A few years later PKG II was cloned from mouse brain (Uhler, 1993), rat intestine (Jarchau et al., 1994) and human (Orstavik et al., 1996). PKG II appears to code for a single protein. The human PKG I and PKG II genes are located on chromosomes 10 (Orstavik et al., 1992) and 4 (Orstavik et al., 1996) respectively. The Drosophila DG1 gene codes for a single protein product, whereas DG2 codes for three T1, T2 and T3 (Kalderon and Rubin, 1989). Sequence comparisons show that bovine PKG I is more similar to DG2 and DG1 than rat PKG II. Phylogenetic analysis indicates that PKG I and DG2 are derived from a common ancestral gene and that the predecessors of PKG II diverged before the appearance of DG 1 in

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evolution. It is speculated that a mammalian branch of DG1 exists that is yet unidentified (Pfeifer et al., 1999). However, a third isoform does not appear to be present in the completed mouse and human genomes.

The conservation of PKG throughout various animal phyla indicates that PKG is an important molecule and the presence of different isoforms indicates that they might have distinctive roles in various physiological processes involving cGMP.

2. PKG Structure

PKG has a very similar structure to PKA. Both belong to the family of serine/threonine kinases that are activated by cyclic nucleotides (Francis and Corbin, 1999; Hanks and Hunter, 1995). The enzymes consist of three major domains, an amino-terminal, a regulatory (R) and a catalytic (C) domain. The regulatory domain contains two cyclic nucleotide binding sites and the catalytic domain consists of the Mg-ATP and peptide binding pockets. The catalytic domain catalyses the transfer of γ phosphate from ATP to a serine/threonine residue of the target protein (Pfeifer et al., 1999). Mammalian PKGs (Gamm et al., 1995; Hofmann et al., 1992), DG1 (Foster et al., 1996), DG2-T1 and DG2-T3 (Kalderon and Rubin, 1989) are dimers, whereas the PKG purified from *Paramecium* (Miglietta and Nelson, 1988), *Tetrahymena* (Murofushi, 1974), and *Dictyostelium discoideum* (Wanner and Wurster, 1990) are monomeric enzymes. It is unclear if this difference in subunit composition is inherent to the enzymes or the result of partial proteolysis of the dimeric enzymes (Hofmann et al., 1992)since partial proteolysis of mammalian PKG can result in a

monomeric enzyme that can be activated by cGMP (Wolfe et al., 1989b).The inactive PKA is a tetramer of two R and two C subunits in which the two R subunits are tightly bound. Binding of cAMP dissociates the holoenzyme into a R2 (cAMP) dimer and two active C subunits. In contrast, the R and C domains of PKG are present on a single polypeptide chain. Binding of cGMP does not dissociate the PKG enzyme to R and C subunits (Pfeifer et al., 1999).

A. Dimerization

The amino terminus of PKG I and II consists of a hydrophobic leucine/Isoleucine zipper motif (a heptad repeat, containing a leucine/isoleucine residue at every first out of seven aa residues), which is important for dimerization of the proteins(Atkinson et al., 1991; Gamm et al., 1995; Landgraf et al., 1990; Vaandrager et al., 1997). Six heptad repeats are present in PKG Iα, 7-8 in 1β and 8-9 in PKG II (Richie-Jannetta et al., 2003). Proteolysis just carboxy-terminal of this domain produces a monomeric PKG that begins just amino-terminal of the autoinhibitory domain (Francis et al., 1996; Monken and Gill, 1980; Wolfe et al., 1989b). *In vitro*, the monomeric enzymes retain the autoinhibition, autophosphorylation, cGMP binding and kinase activity of the dimeric PKGs (Richie-Jannetta et al., 2003). Dimerization has been shown to increase sensitivity to cGMP activation of PKG Iβ (Richie-Jannetta et al., 2003). Dimerization has also shown to be important for binding of probable anchoring proteins and substrates such as troponin T (Yuasa et al., 1999), GKAP 42 in germ cells (Yuasa et al., 2000), and the myosin-binding subunit of myosin phosphatase (Surks et al., 1999) for PKG 1α and troponin T and inositol 3-phosphate receptor-associated PKG substrate (Ammendola

et al., 2001) for PKG 1β. Dimerization in PKG I seems to be important for proper enzyme substrate complex formation. Influence of dimerization on PKG II properties has not been well studied.

B. Lipid modification and its importance

PKG I and PKG II are acetylated and myristoylated respectively (Takio et al., 1984; Vaandrager et al., 1996). Vaandrager et al. did not find any evidence of palmitoylation or other lipid modification of PKG II (Vaandrager et al., 1996). PKG I is a soluble protein (Jarchau et al., 1994; Lohmann et al., 1997). PKG II in contrast is a membrane bound enzyme and myristoylation has been shown to be important for membrane targeting of PKG II (Vaandrager et al., 1996).

a. N-Myristoylation

Protein N-myristoylation (N-myr) is an irreversible co-translational modification that occurs following removal of the initiator methionine residue by cellular methionylaminopeptidases (Towler et al., 1987; Wolven et al., 1997). It refers to the covalent attachment of myristate, a 14-carbon saturated fatty acid, to the N-terminal glycine of eurkaryotic and viral proteins (Farazi et al., 2001). The step is catalyzed by the enzyme myristoyl CoA:protein N-myristoyltransferase (Towler et al., 1987). N-myr can also occur post-translationally as in the case of the pro-apoptotic protein BID where proteolytic cleavage by caspase 8 reveals a "hidden" myristoylation motif (Zha et al., 2000). N-myr promotes weak and reversible

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protein-membrane interactions (Murray et al., 1997; Peitzsch and McLaughlin, 1993). N-Myr of PKG II has been shown to be responsible for PKG II to preferentially phosphorylate the CFTR Cl channel in an intestinal cell line (Vaandrager et al., 1998). In the same study PKG Iβ, which is a soluble protein, was unable to activate the CFTR channel, however, a chimaera with the first 29 amino acids of PKG II attached to the N-terminus of PKG Iβ protein was able to activate the CFTR channel in the intestinal cell line (Vaandrager et al., 1998), signifying the importance of targeting in specific phosphorylation events. N-myr was shown to be important for targeting of a post-synaptic protein PSD-Zip70 to apical plama membranes of microvilli in Maldin-darby canine kidney cells and for proper targeting in hippocampal neurons in culture (Konno et al., 2002). Dresbach et al showed that Bassoon, a presynaptic particle web protein, requires N-myr for targeting to membrane-bound synaptic organelles (Dresbach et al., 2003).

PKG II is myristoylated and appears to be present in neuronal processes (de Vente et al., 2001). We tested the hypothesis that a) N-myr affects localization of PKG II in heterologous systems such as COS and HEK293 cells; b) N-myr is important for targeting of PKG II in neurons.

C. Autoinhibition and Autophosphorylation

In PKA and PKG autoinhibition is dependent upon the electrostatic interaction between positively charged residues of the pseudosubstrate sequence within the regulatory domain and negatively charged residues within the catalytic cleft (Francis and Corbin, 1994; Lincoln

and Corbin, 1978). Ser[64] and Ser[79] in the autoinhibitory pseudosubstrate site in PKG Iα and 1β, respectively, were shown to be important for autoinhibition and mutation of these residues led to a decrease in cGMP binding affinity (Busch et al., 2002). In PKG II amino acids Lys[122], Arg[118] and Arg[119] were shown to be responsible for autoinhibition (Taylor et al., 2002). Cyclic nucleotide binding leads to autophosphorylation of specific amino acids in the pseudosubstrate motif of PKA, and decreases the rate of association between the R and C subunits of the type II isoform of PKA (Scott and Mumby, 1985). The decrease in rate of association is thought to be due to electrostatic repulsion between the acidic residues within the catalytic cleft and the negatively charged phosphates incorporated into the autophosphorylated regulatory domain (Taylor et al., 2002). PKG I exhibits similar autoinhibitory characteristics of PKA. Some amino acids responsible for autoinhibition of PKA are conserved in PKG I and II (Taylor et al., 2002). Possible sites for autophosphorylation in PKG Iα are Ser[1], Ser[50], Thr[58], Ser[64], Ser[72] and Thr[84] {Monken, 1980 #210;Takio, 1984 #195;Aitken, 1984 #211} and in PKG Iβ Ser[63] and Ser[79] (Smith et al., 1996; Wolfe et al., 1989a). Autophosphorylation of PKG Iα and Iβ have been shown to increase the basal kinase activity and increase its affinity for cGMP (Busch et al., 2002). In addition PKG Iα also undergoes autophosphorylation in the presence of cAMP, which increases its affinity for cAMP. The increase in affinity for cAMP, however, does not occur when PKG Iα is activated in the presence of cGMP (Aitken et al., 1984; Francis et al., 1996). A similar effect has been demonstrated for PKG Iβ, in which autophosphorylation in the presence of both cAMP and cGMP leads to an increase in basal activity of the enzyme and increases the affinity for both cyclic nucleotides (Smith et al., 1996). In PKG II the amino acids Ser[110], Ser[114], and at a slow rate Ser[126], Thr[109] or Ser[117], all located in the autoinhibitory

region of PKG II, were shown to be autophosphorylated after cGMP treatment *in vitro* (Vaandrager et al., 2003). Mutations of Ser^{110} and Ser^{114} did not alter either kinase activity or affinity for cGMP, however, mutation of the slowly autophosphorylated Ser^{126} generated a constitutively active PKG II (Vaandrager et al., 2003). Autophosphorylation of PKG II has been shown in the presence of cAMP as well, however, at a 100-fold higher concentration of cAMP (de Jonge, 1981). In contrast the autophosphorylation of PKG Iβ occurs at just a 2-fold higher concentration of cAMP to cGMP (Smith et al., 1996). In the above studies a recombinant PKG II which was non-myristoylated was used. It has, however, been shown that lack of N-myr does not affect specific activity or affinity for cGMP (Vaandrager et al., 1996). Apart from the above mentioned differences between PKG I and II, native PKG Iα possesses a significantly higher affinity for cGMP and β-phenyl-1-N2-etheno-cGMP than recombinant PKG II. In contrast the Sp- and Rp- isomers of 8-(4-chloro-phenylthio)-guanosine-3',5'-cyclic monophosphorothioate demonstrated selectivity toward PKG II (Gamm et al., 1995). **PKG I and PKG II thus have very different physical and biochemical properties and it is plausible to assume that this could result in distinct functions.**

Figure 1: Functional domains of PKA and PKG.

The linear arrangement of functional domains of PKA and PKG is shown (Francis and Corbin, 1999). The amino terminal region has the dimerization domain followed by the autoinhibitory region and autophosphorylation sites (see text for detail). This region is followed by two cAMP sites in PKA regulatory domain (A) and cGMP sites in PKG I (C) and PKG II (D), followed by the catalytic domain containing the Mg^{2+}-ATP binding region and substrate bindng region. The region of the antigenic peptide used for generating an antibody against PKG II is shown in D.
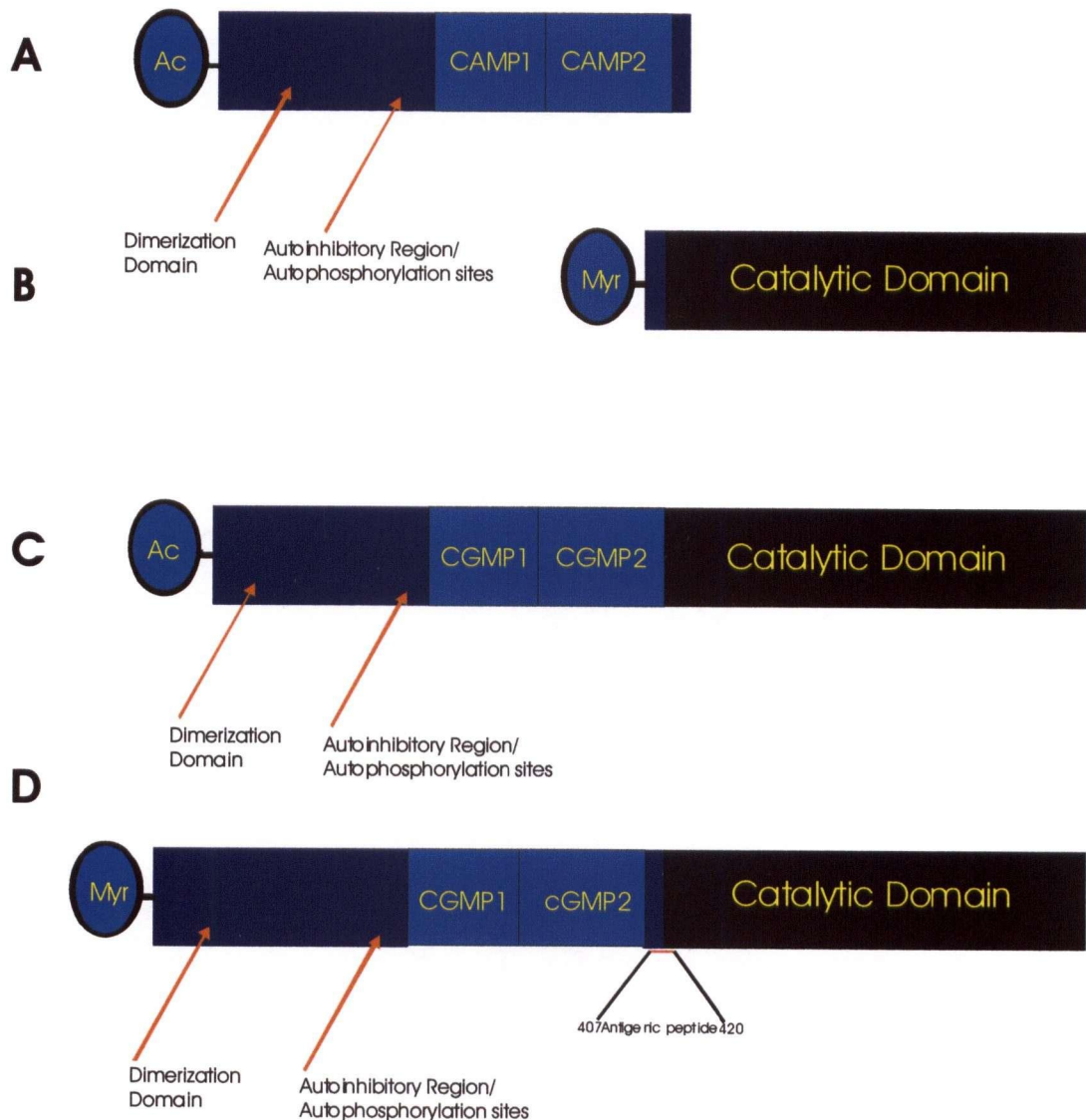
3. PKG Expression

A. Expression in C. elegans and Drosophila

The *C. elegans* PKG CGK-1C has been shown to be strongly expressed in the ventral nerve cord and in several other neurons including PQR. It is also expressed in pharyngeal marginal cells, body muscle, intestine, vulval muscles, and spermatheca (Stansberry et al., 2001).

Both DG1 and DG2 are expressed during *Drosophila* embryonic development (Kalderon and Rubin, 1989). Foster et al. showed that embryonic DG1 is temporally restricted to stage 13 embryos and is confined to the cephalic region and to the amnioserosa (Foster et al., 1996). In the adult fly, DG1 transcript was found in head tissue, such as optic lobes and proximal cortex (Foster et al., 1996; Kalderon and Rubin, 1989). Northern blot analyses showed that transcripts of DG2 kinase are expressed in adult head and body tissues of the fly (Kalderon and Rubin, 1989)

B. Expression of mammalian PKGs

a. PKG I

The highest concentration of PKG I is found in smooth muscle, platelets and cerebellum (Keilbach et al., 1992; Lohmann et al., 1981; Waldmann et al., 1986). Presence of PKG I has also been reported in the hippocampus (Kleppisch et al., 1999), dorsal root ganglia (Qian et al., 1996), neuromuscular endplate (Chao et al., 1997) and cells of the kidney vasculature (Joyce et al., 1986). However, El-Husseini et al did not find any PKG I expression in

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hippocampus, but found a small cluster of neurons in the compact portion of the dorsomedial nucleus of the hypothalamus in addition to Purkinje cell staining in the cerebellum (El-Husseini et al., 1998). Lower levels of PKG I have been reported in vascular endothelial cells (MacMillan-Crow et al., 1994), and immune cells {Pryzwansky, 1995 #223}. PKG I is also found in the bone in growth plate chondrocytes (Pfeifer et al., 1996) and osteoclasts (Van Epps-Fung et al., 1994). Using PKG Iβ specific antibodies in bovine and rat tissues it was shown that PKG Iβ is highly expressed in the uterus, aorta, and trachea but not in lung, heart and cerebellum (Keilbach et al., 1992), which have been shown to have high concentrations of PKG Iα.

b. PKG II

PKG II is predominantly localized in the brain (El-Husseini et al., 1998), intestinal brush border (Markert et al., 1995), proximal convoluted tubules of the kidney (Gambaryan et al., 1996), the ciliary epithelium of the epidydimis, bone and the lung (Pfeifer et al., 1999). Using *in situ* hybridization El-Husseini showed high levels of PKG II mRNA in the Olfactory bulb, the outer layers of cerebral cortex, pyriform cortex, lateral amygdala, the septum, the thalamus, the superior colliculus, the locus ceruleus, the pontine nuclei and the nucleus of the solitary tract (el-Husseini et al., 1995; El-Husseini et al., 1998). De Vente et al showed using western blot analyses and immunohistochemistry that PKG II is present in at least 38 different regions of the brain including cerebellar cortex (de Vente et al., 2001). They also found expression of PKG II in oligodendrocytes and astrocytes. De Vente et al. also showed the presence of PKG II in some pyramidal neurons in the hippocampus and

dendrites of cerebellar purkinje cells. PKG II expression in cerebellum has not been observed by others (el-Husseini et al., 1995; El-Husseini et al., 1998). De Vente et al did not find PKG II co-localized with cGMP in many regions of the brain. Their study also did not find significant cell body staining of PKG II, which did not coincide with the mRNA localization observed by El-Husseini et al (de Vente et al., 2001; el-Husseini et al., 1995; El-Husseini et al., 1998). This raises a few doubts about the specificity of the antibody used. Further studies with different antibodies need to be done to characterize the distribution of PKG II.

PKG I and PKG II have a very different expression pattern, with PKG II having a much wider expression in the brain compared to PKG I. This indicates that PKG II is most likely to be the candidate for mediating most of the cGMP effects in the brain that require phosphorylation.

Although it is now known to a certain extent what tissues and what regions within those tissues express PKG I and PKG II, the subcellular localization of PKG I and PKG II is still unclear. Is it present at the synapse? Is it presynaptic or postsynaptic in location or both? There are still such unanswered questions and one of the objectives of this thesis research was to define the subcellular distribution of PKG I and PKG II.

4. PKG substrates

Initial studies on PKG substrate specificity were made using both protein and peptide substrates that were phosphorylated *in vitro* by homogeneous preparations of PKG and PKA

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(Lincoln and Corbin, 1977), which indicated that the same primary amino acid sequence (Arg-Arg-X-Ser(P)/Thr(P) was sufficient for substrate recognition by both PKA and PKG, consistent with a close evolutionary relationship between the two kinases (Corbin et al., 1990). Later proteins that were phosphorylated preferentially, but not exclusively by PKG were found like histone H2B (Glass and Krebs, 1982), bovine lung cGMP-binding cGMP-specific phosphodiesterase (cG-BPDE) (Thomas et al., 1990a; Thomas et al., 1990b). Colbran et al. showed that a Phenylalanine in peptide substrates confers selectivity between PKA and PKG (Colbran et al., 1992). Butt et al showed that Ser[157] on Vasodialator-stimulated phosphoprotein (VASP) is preferred more by PKA and Ser[239] by PKG (Butt et al., 1994). **This shows that although PKA and PKG are evolutionarily similar they have substrates that are phosphorylated preferentially, which indicates that PKG might have specific in vivo substrates and distinct functions from that of PKA.**

PKG I has been shown to preferentially phosphorylate a broad range of proteins *in vitro*, however, phosphorylation of very few of these proteins have been shown to occur *in vivo*. The substrates of PKG I known so far are; 1) IP3 receptor (Komalavilas and Lincoln, 1994; Komalavilas and Lincoln, 1996) and phospholamban (Raeymaekers et al., 1990), which are implicated in SMC relaxation; 2) The vasodilator-stimulated phosphoprotein and vimentin, which are involved in platelet aggregation and neutrophil activation respectively (Aszodi et al., 1999; Pryzwansky et al., 1995); 3) thrombaxane A_2 receptor, the activation of which was inhibited in platelets (Wang et al., 1998); 4) the large conductance, voltage-dependent, and calcium-sensitive K+ channel, Hslo (Alioua et al., 1998); 5) the L-type Ca^{2+} -channel (Jahn et al., 1988) and the large conductance Ca2+-activated K+ channel, *cslo-α* on Ser[1072] in

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HEK-293 cells (Fukao et al., 1999), which when phosphorylated are thought to regulate VSM tone and cardiac contractility; 6) Phospholipase A_2, implicated in intestinal smooth muscle relaxation (Murthy and Makhlouf, 1999); 7) a tyrosine hydroxylase, the activity of which was increased by PKG I in intact bovine chromaffin cells (Rodriguez-Pascual et al., 1999); 8) myosin binding domain of myosin light chain phosphatase, which plays a role in SMC relaxation and vasodilation (Surks et al., 1999); 9) p38 MAPK in 293T Fibroblasts (Browning et al., 2000), and in the brain; 1) Septin 3 in nerve terminals, where PKG I mediated phosphorylation is thought to affect Septin 3 localization, hence its function (Xue et al., 2004), and. 2) G-substrate in the purkinje cells, through which it inhibits protein phosphatase-1 {Endo, 1999 #170;Hall, 1999 #176}. PKG I can phosphorylate DARP-32, a dopamine- and cAMP regulated phosphoprotein, *in vitro* and cGMP has been shown to stimulate DARP-32 phosphorylation in the substantia nigra. PKG Iα is thought to be responsible for phosphorylation of most of the above mentioned substrates. PKG 1β has been indirectly shown to phosphorylate cytoskeletal and contractile proteins such as myosin light chain, calponin, desmin, connexins and regulate vascular remodeling and neoangiognesis {Lincoln, 1998 #282}.

In contrast only one specific substrate of PKG II is known, which is the CFTR Cl- channel. Only PKG II and not PKG I can phosphorylate CFTR *in vivo*. PKG II regulates intestinal Cl- and water secretion by phosphorylating CFTR in the intestinal epithelial cells (Vaandrager et al., 1998; Vaandrager et al., 1997).

It has been shown that a peptide substrate derived from histone f_{2B} had much higher specificity for PKG Iα than for PKG II, whereas a peptide based upon CREB phosphorylation site exhibited a greater selectivity for PKG II. IP$_3$Rtide, derived from IP$_3$R and kemptide, derived from pyruvate kinase did not show any diference in selectivity for either PKG Iα or II. However, BPDEtide, derived from bovine cG-BPDE, exhibits a two fold lower selectivity to PKG II than to PKG I.

The data above indicate that it is quite likely that PKG I and PKG II have unique substrates and possibly mediate different cGMP effects.

A. Unknown substrates of PKG in the brain

Initial studies reported that the brain is lacking in endogenous PKG substrates (Walaas et al., 1989). In synaptosomes, only a single 60 kD protein whose phosphorylation was stimulated by cGMP has been reported in human postmortem brain (Boehme et al., 1978). It is thought that the reasons for lack of endogenous substrates of PKG were related to two technical factors. The endogenous level of PKG in most brain regions was insufficient to detect substrate phosphorylation, and the background activity of other protein kinases was too high, masking detection of potential PKG substrates (Wang and Robinson, 1997). After overcoming this problem Wang and Robinson detected >40 relatively specific PKG substrates in the brain. They found 10 proteins in the nerve terminals indicating a neuronal localization of the probable substrates (Wang and Robinson, 1995). El-Husseini et al. showed PKG mediated phosphorylation of a number of proteins from thalamic extracts (El-

Husseini et al., 1998). This indicates that a number of PKG substrates exist in the brain and mediate specific signaling via cGMP. **As PKG II is much more widely expressed than PKG I in the brain, most of these substrates are likely to be PKG II substrates. Hence, a main goal of the thesis was to find the function of PKG II in the brain as it is likely to be the major downstream effector of NO.**

a. Is yeast two-hybrid a good system to use to find partners of PKG II?

We initially used the yeast two-hybrid system (Y2H) (Fields and Song, 1989) to find probable substrates and anchoring proteins of PKG II. The catalytic domain of PKG II was expressed in yeast along with a brain cDNA library to find probable substrates. We also used the N-terminus (first 100 aa) of PKG II as bait to find anchoring proteins. However, after screening ~5 million clones in each category we failed to find any interactions. All the required controls were done to make sure the protein was expressed in yeast and the system was working. We realized that since cyclic nucleotide kinases have very high rates of reaction, the transient interaction with the substrate may not be enough to activate the reporter gene expression. So we made a catalytically mutant (D594N) (Yoo and Hamburger, 1998) form of PKG II and used it as a bait in the Y2H system to find interactions. Even that did not yield any positives, neither did a FL wt PKG II bait. It is possible that PKG II, which is myristoylated, may not attain the proper conformation due to improper co/post-translational modification in yeast and hence interaction might not occur. Proteins called Receptors for activated C-kinase (RACKs) only bind activated PKC and enhance its activity (Mochly-Rosen et al., 1991). Activation of PKG II by cGMP may be required for interaction

to occur and so far no cGMP presence has been reported in yeast, which could be a reason for the lack of positives in the Y2H system. It is also possible that proteins interacting with PKG II are developmentally regulated or very weakly expressed. It appears that the Y2H is not the best system to use to find partners of PKG II.

VI. NO going back, NO/NPs to cGMP to PKG and beyond.

1. Role in regulation of gene expression

NO regulates vascular tone via cGMP and PKG causing smooth muscle relaxation in a number of ways, including lowering of intracellular Ca2+ and inhibition of RhoA-dependent Ca2+ sensitization of contraction (Lohmann et al., 1997; Sauzeau et al., 2000). cGMP, however, also positively regulates RhoA expression in vascular smooth muscle cells (VSMC) (Sauzeau et al., 2003). When exposed to NO and cGMP analogues for a long time, RhoA expression is increased by both increase in *rhoA* transcription and RhoA protein stability because of PKG phosphorylation. The PKG mediated increase in transcription is associated with increased CREB and ATF-1 phosphorylation (Sauzeau et al., 2003).

cGMP and PKG are also important for VSMC differentiation and phenotypic modulation. Synthetic VSMCs transfected with constitutively active PKG or FL PKG (activated with cGMP) restored a more contractile phenotype with fusiform morphology, increased expression of smooth muscle myosin heavy chain-2 (SM-MHC2), SM α-actin and calponin protein, and decreased expression of osteopontin, thrombospondin, and FGF receptors

(Boerth et al., 1997; Dey et al., 1998; Lincoln et al., 2001; Lincoln et al., 1998). Recently it was shown using cDNA micro-array analyses to compare PKG transfected and control transfected late passage VSMCs, that >100 transcripts could be up or down regulated more than three fold by cGMP/PKG (Pilz and Casteel, 2003).

It has been shown that cGMP/PKG can have anti-proliferative effects on VSMC, mesangial cells, and various fibroblasts by inhibiting growth factor-induced extracellular signal-regulated kinase (Erk-1/2) activity, increased expression of MAP kinase phosphatase-1 (MKP-1) (Hutchinson et al., 1997; Suhasini et al., 1998; Yu et al., 1997), modulation of cell cycle-associated genes, and reduction of ET-1 synthesis (Fujisaki et al., 1995; Mitsutomi et al., 1999). cGMP has also been shown to increase proliferation of endothelial cells increasing Erk-1/2 activity possibly by increased production of vascular endothelial growth factor (VEGF) (Doi et al., 2001; Hood and Granger, 1998; Parenti et al., 1998; Zhang et al., 2003).

cGMP analogues can prevent apoptosis of neurons that is induced by prolonged treatment of NOS and sGC inhibitors. The protective effect of cGMP is associated with increased CREB phosphorylation and increased mRNA and protein expression of the apoptosis inhibitor Bcl-2 (Ciani et al., 2002). cGMP/PKG have been shown to protect neuronal cells from apoptosis during growth factor deprivation by increasing expression of the oxidative stress-related proteins thioredoxin and thioredoxin peroxidase (Tpx-1), which leads to increased Bcl-2 expression (Andoh et al., 2003).

Transcription factors can be regulated by cGMP directly by inducing phosphorylation by PKG or by increasing expression of short-lived proteins. Transcription factors that could be phosphorylated in a cGMP-dependent manner include the cAMP response-element (CRE)-binding protein CREB in neuronal cells (Ciani et al., 2002; Lu et al., 1999), activating transcription factor-1 (ATF-1) (Sauzeau et al., 2003), and the multifunctional transcription factor TFII-I (Casteel et al., 2002). cGMP also regulates expression of transcription factors such as the AP-1 family proteins c-Fos and JunB (Pilz et al., 1995; Thiriet et al., 1997), the early growth response gene Egr-1(Cibelli et al., 2002; Esteve et al., 2001; Thiriet et al., 1997; Yamashita et al., 1997), and the growth arrest–specific homeobox gene GAX . Increase in intracellular cGMP leads to increased CREB Ser^{133} phosphorylation in VSMCs, neuronal cells, and PKG-transfected Baby Hamster Kidney (BHK) cells, but not in PKG-deficient BHK cells (Ciani et al., 2002; Gudi et al., 2000; Lu et al., 1999; Sauzeau et al., 2003). Some researchers have shown nuclear translocation of PKG in neuronal cells, neutrophils, macrophages, and some embryonic smooth muscle cells (Gudi et al., 2000; Gudi et al., 1997; Pryzwansky et al., 1995; Wang et al., 1999; Wyatt et al., 1991), while others have found no evidence of PKG nuclear translocation in primary VSMCs, HEK293, and CV-1 cells, or observed nuclear PKG only in a minority of the cell population {Collins, 1999 #135;Browning, 2001 #136;Feil, 2002 #137}.

cGMP could indirectly regulate transcription factors by modulating upstream signal transduction pathways, which include cGMP regulation of an inhibitor of NF-[kappa]B, and inhibition of calcineurin signaling to NF/AT and of RhoA signaling to SRF. cGMP can regulate the activity of multiple transcription factors, including ternary complex factor

(TCF), CREB, ATF-2, and c-Jun through activation or inhibition of MAP kinase pathways (Hazzalin and Mahadevan, 2002; Pilz and Casteel, 2003).

Together, this data indicates that cGMP/PKG plays a very important role in gene expression, apoptosis, cell growth, and differentiation not only in cardiovascular system but also in neurons and other cell types.

2. Role in modulation of neurotransmitter release and synaptic plasticity

A. Neurotransmitter release

A number of studies have reported that NO is important in neurotransmitter release. Release of L-glutamate and norepinephrine upon NMDA receptor (NMDAR) activation in synaptosomal preparations was shown, and this effect was mediated by NO (Montague et al., 1994). Superfusion with the NOS inhibitor, N^G-nitro-L-arginine in the basal forebrain of conscious rats diminishes the release of acetylcholine (Prast and Philippu, 1992). Sorkin et al. showed activation of NMDA receptors results in extracellular release of glutamate and NO (Sorkin, 1993). Inhibition of NOS blocked evoked increases in extracellular glutamate (Sorkin, 1993) and this effect was shown to be cGMP mediated (Sistiaga et al., 1997). There is evidence for NO mediated modulation of neurotransmitter release of dopamine (Hanbauer et al., 1992) and GABA (Li et al., 2004). cGMP/protein kinase G pathway has been shown to potentiate glutamatergic transmission induced by NO in immature rat rostral ventrolateral medulla neurons in vitro (Huang et al., 2003). The NO/cGMP pathway is not only important for neurotransmitter release, but also for regulation of synaptic vesicle endocytosis (Micheva

et al., 2003). In all these cases NO is thought to act as a retrograde messenger and activate sGC and PKG at the presynaptic terminal. Modulation of neurotransmitter release is important as it plays a role in synaptic plasticity. Most of the evidence mentioned above was obtained using activators and inhibitors against NOS, sGC and PKG. **Although it is clear that the NO/cGMP/PKG pathway is involved, it is still unclear how these effects are mediated by PKG.**

B. NO Hippocampal LTP

Hippocampus is responsible for the formation of declarative and spatial memory (Milner et al., 1998). Associative LTP has been shown in pyramidal cells of the CA1 region of hippocampus (Shors and Matzel, 1997). The Schaffer collaterals release glutamate which activates NMDAR. This allows Ca^{2+} to enter the cell and activate $Ca^{2+}CaM$ (Wang and Kelly, 1995), which in turn activates NOS and causes release of NO from postsynaptic CA1 pyramidal cells (Susswein et al., 2004). LTP is thought to be initiated postsynaptically, although there is evidence that it is maintained presynaptically (Arancio et al., 1996). However, others have argued against a presynaptic locus of LTP expression (Luscher et al., 2000; Malenka and Nicoll, 1999). Schuman and Madison proposed that NO can act as a retrograde messenger to send the signal back to the presynaptic terminal after LTP initiation on the postsynaptic side (Schuman and Madison, 1994). Of the two forms of NOS (nNOS, eNOS) expressed in the hippocampus, knockout of either isoform alone does not block LTP expression, but double mutants in which both forms are non-functional show a decreased LTP in some areas of the CA1 region but not others (Son et al., 1996). There is also evidence

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that NO has no effect on either LTP in the hippocampus (Bannerman et al., 1994b; Cummings et al., 1994; Murphy and Bliss, 1999) or on memory affected by the hippocampus (Bannerman et al., 1994a; Tobin et al., 1995).

cGMP and PKG have also been implicated in regulation of LTP. Blocking PKG has been shown to block LTP and selective activators of PKG can produce LTP, implicating the cGMP second messenger pathway (Son et al., 1996; Zhuo et al., 1994a). In cultured hippocampal neurons, intracellular injection of PKG blockers in the presynaptic neuron, but not in the postsynaptic neuron prevents LTP, indicating a presynaptic involvement (Arancio et al., 2001). However, there are also reports that blocking the cGMP pathway, either via pharmacological agents or knocking out genes, fails to block LTP (Schuman et al., 1994; Selig et al., 1996). Kleppisch et al., found that LTP was not altered in PKG I knockout or PKG II knockout or a double knockout of PKG I and II mice (Kleppisch et al., 1999). These studies found that the effect is probably mediated by ADP-ribosylation of proteins, as using pharmacological inhibitors of ADP-ribosylation blocked LTP (Kleppisch et al., 1999; Schuman et al., 1994; Selig et al., 1996). Other studies showed that PKG activates phosphodiesterases, which degrade cGMP, and thereby lowers its concentration and this effect is necessary for LTP (Monfort et al., 2002). The contradictory evidence regarding involvement of the cGMP/PKG pathway could be because of the high sensitivity of cGMP analogues to experimental conditions {Son, 1998 #283} or possibly because effects of NO on LTP could differ considerably between different strains and species, at different stages of development, and as a result of small differences in protocols used (Blackshaw et al., 2003; Holscher, 2002)

Most of the above mentioned studies argue for a role of NO on the presynaptic side. There is also evidence that NO can act post-synaptically, either by enhancing LTP in some cases or by suppressing it in others (Ko and Kelly, 1999; Murphy and Bliss, 1999). Ko and Kelly showed that extracellular application of the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) or postsynaptic co-injection of L-NAME with Ca(2+)/CaM blocked Ca(2+)/CaM-induced synaptic potentiation. Murphy and Bliss showed the opposite effect using flash photolysis of a caged form of NO. They found that photolytically released NO (1-4.5 microM) from bath applied caged NO reduced the magnitude of long-term potentiation (LTP) in a concentration-dependent manner. **However, the postsynaptic effects downstream of NO have not been studied in detail.**

C. NO Cerebellar LTD

NO is released by parallel fibre activity and climbing fibre activity causes an increase in Ca^{2+} entry into Purkinje cells. NO acts through sGC and causes an increase in cGMP, which in turn activates PKG and causes initiation of LTD (Lev-Ram et al., 1997). NO was found to induce LTD when paired with low-frequency stimulation of 0.25 Hz, a frequency that alone did not induce LTD (Zhuo et al., 1994b). NOS inhibitors also blocked LTD induction in the CA1 region of hippocampus (Izumi and Zorumski, 1993) and the dentate gyrus (Wu et al., 1997). The same effect was observed when sGC and PKG inhibitors were used, indicating that NO acts through the cGMP/PKG pathway to induce LTD in the dentate gyrus (Wu et al., 1998). Calabresi et al. showed that the NO/cGMP/PKG pathway is also involved in LTD

induction in the corticostriatal pathway (Calabresi et al., 1999). However, Glaum et al. showed that NO donors do not induce LTD (Glaum et al., 1992). Another group found that in Purkinje cells NOS inhibition or hemoglobin did not prevent depression of glutamate sensitivity nor could it be mimicked by an exogenously applied NO donor (Linden and Connor, 1992).

All this evidence suggests an important role for the cGMP/PKG pathway in regulating synaptic plasticity, however, there is still a lot of controversy over the role of NO in modulation of LTP and LTD. It is also still unclear as to how exactly the cGMP/PKG pathway is involved in regulating plasticity, especially on the postsynaptic side.

D. Possible role in regulation of filopodia/spine morphology

Post synaptic changes that have been associated with LTP and LTD include phosphorylation of glutamate receptors (Barria et al., 1997; Lee et al., 2000; Nicoll and Malenka, 1999), insertion of receptors in "silent" synapses (Liao et al., 1995), modification of electronic properties and dendritic spine shape (Muller et al., 2000). Modulation of the plasma membrane shape and composition regulate outgrowth of processes, axonal development, dendritic branching and synaptogenesis (Jontes and Smith, 2000). In non-neuronal cells, differential regulation of membrane flow can result in the formation of processes such as microspikes, lamellopodia, and filopodia (Wood and Martin, 2002). Dendritic filopoida are thought to be precursors for developing synapses (Dailey and Smith, 1996; Hering and Sheng, 2001). The NO/cGMP pathway has shown to be important for regulation of growth

cone filopodia (Van Wagenen and Rehder, 1999), and neurite outgrowth in mouse hippocampal neurons and PC12 cells (Hindley et al., 1997). The change in spine shape associated with LTP has been shown to involve an increase in F-actin (Lisman, 2003). Ena/VASP proteins, which are specific substrates of PKG and PKA, have been shown to play an important role in linking signaling pathways to remodeling of the actin cytoskeleton [for review see (Kwiatkowski et al., 2003)]. cGMP inhibits collagen-induced platelet aggregation, which requires dynamic actin reorganization followed by cell shape change {Aszodi, 1999 #347}. PKG has been shown to mediate platelet aggregation through VASP {Aszodi, 1999 #347} and phosphorylation of VASP by PKA has been shown to reduce the ability of VASP to promote in vitro nucleation probably by reducing VASP binding to G-actin (Harbeck et al., 2000; Lambrechts et al., 2000; Walders-Harbeck et al., 2002). NO has been implicated in agrin-induced postsynaptic differentiation at the neurosmuscular junction and has been shown to act through cGMP (Godfrey and Schwarte, 2003). **All this data leads us to hypothesize that the cGMP/PKG pathway plays an important role in regulation of filopodia/spine morphology.**

3. PKG and behaviour

PKG has been shown to be important for regulation of complex behaviours. Genetic studies in *Drosophila* have shown that the *for* locus, which encodes a PKG isoform, influences heritable patterns of larval foraging behavior. Animals carrying a *"rover"* allele (*for^R*) move long distances while feeding, while insects homozygous for the *"sitter"* allele (*for^S*), a naturally occurring variant that has less PKG, are relatively inactive in the presence of food

(Osborne et al., 1997). Using behavior genetic analyses it was shown that PKG in *C. elegans* is important for normal motility (Stansberry et al., 2001), olfactory adaptation (L'Etoile et al., 2002) and for regulating multiple developmental and behavioral processes including orchestrated growth of the animal and the expression of particular behavioral states (Fujiwara et al., 2002). This indicates PKG plays an important role in model organisms such as *Drosophila* and *C. elegans*, however, the role of PKG in the mammalian nervous system is still unclear.

4. Statement of Aims

We hypothesize that PKG II is the major candidate for mediating cGMP effects in the brain. Our first aim was to confirm the hypothesis stated above by showing that PKG II has a wide expression in the brain using a PKG II specific antibody. We also hypothesize that PKG II is present in the synapse and N-myr is important for targeting of PKG II to the synapse. We wanted to test this hypothesis by showing the presence of PKG II, using PKG II specific antibody, in synaptic fractions from the brain and co-localization of PKG II with synaptic markers in hippocampal neurons in culture. Our aim was also to show that N-myr is important for proper targeting of PKG II in neurons and heterologous cells by using a non-myristoylation mutant form of PKG II. Finally we wanted to test the hypothesis that PKG II plays an important role in regulation of synaptogenesis in hippocampal neurons in culture by overexpressing a dominant negative form of PKG II.

In this thesis, I will be presenting evidence for the synaptic localization of PKG II, the importance of N-myr on PKG II targeting in neurons and the role of PKG II in regulation of synaptogenesis in hippocampal neurons in culture.

Materials and Methods

I. Animal Care

All animal procedures were in strict accordance with the guidelines of the Canadian Council of Animal Care. All animals were obtained from the Animal Care Centre of UBC.

II. PKG II Antibody Generation and Purification

1. Antibody generation

PKG II antibody was made by Affinity Bioreagents, Inc. The peptide NH_3–TLNRDDEKRHAKRS–COOH corresponding to amino acids 407 – 420 of PKG II was used to generate antibodies against PKG II. The sequence was used in the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) to make sure that there were no sequences similar to it in other proteins. KLH was used as a carrier protein. Pre-Immune serum was collected prior to immunization. Four injections (boosts) of the peptide antigen were given to two rabbits once every 20 days. The first bleed was collected ten days after the third boost, the second and third bleeds were collected 10 and 14 days after the final boost respectively. A fifth and final boost was given a month after the collection of the third bleed. The final bleed, exsanguination bleed, was collected 10 days after the fifth boost. The rabbits were sacrificed after the exsanguination bleed was collected. The pre-immune serum and serum were stored at -80° C.

2. Antibody purification

A. Material

SulfoLink® Kit, PIERCE, Cat. No. 20405

B. Method

Sulfolink® Columns were used to purify the antibodies. 10 mg of peptide (prepared and supplied by Affinity Bioreagents, Inc.) were first reduced using Sulfolink® Reductant containing 2-mercaptoethylamine). The mixture was incubated at 37^0 C for 1.5 hours. Excess 2-mercaptoethylamine was removed using D-Salt® Polyacrylamide Plastic Desalting Column. The reduced protein mixture was applied to the Sulfolink® Coupling Gel column consisting of immobilized iodoacetyl on a crosslinked agarose support. The columns were incubated at room temp. for 30 min. The columns were washed with Sulfolink® Coupling Buffer. One mL of serum was applied to the antigen-coupled column. One mL of sample buffer was applied to the column after the serum entered the gel bed. The column was incubated at room temp. for one hour. The column was washed with 16 mL of sample buffer. One mL fractions of the antibody were eluted using glycine buffer at pH 2.5. The one mL fractions were neutralized by adding 50μL of 1M Tris, pH 9.5. Aliquots of the affinity purified antibodies were stored at -20° C.

III. RNA Extraction

Total RNA was extracted using the single step RNA extraction method as described (Chomczynski and Sacchi, 1987). Brain was dissected from young adult *Rattus norvegicus*

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and homogenized in 1 mL of solution A (4 M guanidinium thiocyanate, 25 mM Na citrate, pH 7.0, 5% sarcosyl, 0.1 M 2-mercaptoethanol) at room temp. After homogenization, 0.1 mL of Na Acetate (pH 4), 1 mL of phenol (water saturated) and 0.2 mL of chloroform/isoamyl alcohol mixture (49:1) were added to the homogenate. The solution was vortexed for 10 sec and cooled on ice for 15 min. It was then centrifuged at 10,000 rpm for 20 min. at 4° C. the aqueous phase (upper layer) was transferred to a fresh tube, mixed with 1 mL isopropanol and incubated at -80° C for 30 min. Samples were then centrifuged at 10,000 rpm for 20 min at 4° C. The precipitate RNA pellet was re-suspended in 300 µL of solution A and RNA was precipitated with 1 vol. of isopropanol. The mixture was centrifuged at 10,000 rpm for 20 min at 4° C to collect the RNA pellet. The pellet was then washed with 70% ethanol, vacuum dried and re-suspended in water and either used immediately or stored at -80° C.

IV. RT Reactions

1. Material

Product	Company	Cat. No.
MMLV reverse transcriptase	Invitrogen	28025-013
RNAguard RNAse Inhibitor	Pharmacia	27-0815-01
dNTPs	Pharmacia	27-2035-01

2. Method

RT reactions were performed similar to the one described earlier (el-Husseini et al., 1994). One µg of total RNA was incubated with 200 U of MMLV reverse transcriptase in a buffer

containing a final concentration of 50 mM Tris (pH 8.3), 74 mM KCl, 3 mM $MgCl_2$, 10 mM

DTT, 5% DMSO, 19 U of RNAse Inhibitor, 0.01 % BSA, 0.25 μg of RT primer

(AGCTACAGCTGAGCTTGAGCTTCAGT$_{20}$), and 0.5 mM of each dNTPs in a final vol. of

10 μL. The reaction mixture was incubated for 2 h at 37° C and either used immediately to

do a PCR or stored at -20° C.

V. PCR

1. Material

Product	Company	Cat. No.
dNTPs	Pharmacia	27-2035-01
VENT Polymerase	New England Biolabs	M0254L
Qiagen Gel Extraction Kit	Qiagen	287-04
T4 DNA Ligase	Pharmacia	27-0870-03

2. Method

PCR was performed in 50 μL reaction containing 1/10 of RT reaction, 60 pM of each primer,

1X PCR buffer, 0.5 mM MgCl2, 200 μM dNTPs, 2 U of VENT DNA Polymerase. The

mixture was overlaid with a drop of mineral oil and then incubated in a GTC-1 genetic

thermal cycler (Scientific Precision) using the following profile: an initial denaturation step

at 94° C for 5 min, then 35 cycles of the following steps, 94° C for 1 min for denaturation,

60° C for 1 min for annealing, and 72° C for 2.5 min for primer extension. The resultant

products were separated by agarose gel electrophoresis, purified using Qiagen gel extraction

kit and digested using appropriate restriction enzymes. The digested products were purified

using Qiagen DNA purification kit and subcloned into the appropriate vector. Ligation reactions were performed in 15 μL vol. containing 1X ligase buffer, 5 U of T4 DNA ligase and 1:3 plasmid vector to cDNA ratio and incubated overnight at 15° C.

VI. Restriction Analysis and Agarose Gel Electrophoresis

1. Material

Product	Company	Cat. No.
HindIII/React Buffer 2	Invitrogen	15207-012
KpnI/React Buffer 4	Invitrogen	15232-010
Hinc II/React Buffer 4	Invitrogen	15206-014
EcoRI/React Buffer 3	Invitrogen	15202-013
1kb ladder	Invitrogen	15615-016

2. Method

Restriction digests were performed to check the integrity of the plasmid vectors, to sub-clone PCR products and to verify prepared DNA constructs. All restriction digests were carried out for at least one h at the appropriate temperature.

Agarose gel electrophoresis was used to verify the size of PCR products, restriction fragments and to estimate the concentration of DNA fragments for sub-cloning. Samples were subjected to electrophoresis on a 1% agarose gel with 0.1 μg/mL of ethydium bromide using 1X TAE as electrophoresis buffer. Samples were loaded with a 1/6th vol. of 6X loading buffer (40% w/v sucrose in water, 0.25% bromophenol blue, 0.25% xylane cyanol). The

mixed samples were loaded into wells of the agarose gel. Electrophoresis was performed at 110V. An ultraviolet transilluminator (300 nm wavelength) was used to visualize DNA. Size was compared to a known 1 kb molecular marker.

VII. Sub-cloning

1. Materials

Product	Company	Cat. No.
DH5 alpha cells	Invitrogen	18265-017
Ampicillin	Sigma	A-9518
Qiagen Mini-prep kit	Qiagen	271-06
Qiagen Maxi-prep kit	Qiagen	121-62
Qiagen Gel Extraction Kit	Qiagen	287-04

2. Method

Fifty μL of chemically competent DH5 α E. coli cells were thawed on ice and transferred to a 13-mL tube containing 250-500 ng of pure plasmid DNA or 1/15 of ligation reaction. The mixture was incubated at 4° C for 15 min and heat shocked at 42° C for 45 sec. The mixture was transferred immediately to 4° C and incubated for 2 min. Five hundred μL of SOC medium were added to each tube and incubated for 1 hr. at 37° C. The cells were centrifuged at 1000g for 5 min, re-suspended in 100 μL of SOC and plated on LB plates containing the selection antibiotic (ampicillin at 100 μg/mL). The plates were incubated at 37° C overnight. Individual colonies were picked and transferred to 13 mL tubes containing 5 mL of LB medium with the selection antibiotic and grown overnight at 37° C. Plasmid DNA was

purified using Qiagen Plasmid Mini or Maxi-prep kit. DNA was sequenced at Nucleic Acid Protein Service (NAPS) Unit, UBC to verify the sequence.

3. Constructs generated for transfection of mammalian cells

A. GFP Fusion Constructs

The mammalian expression vector GW1 containing the sequence expressing GFP (gift from Dr. David Bredt's lab) was used for subcloning all of the PKG II constructs (See Fig. 2). All constructs were subcloned into the HindIII/KpnI sites of GFP-GW1 vector. Primers were ordered from either Genset Oligos or NAPS Unit at UBC. The stop codon was omitted from the reverse sequence in order to make a C-terminal GFP fusion protein. All the constructs were sequenced at the NAPS Unit at UBC to verify that the PKG II sequence was correct and in frame with the sequence expressing GFP. Western blot was performed using GFP antibodies on lysates from HEK-293 cells expressing the various constructs to make sure the fusion proteins were expressed (See Fig. 3).

a. FL wt PKG II

The forward primer 5'-GCTAGC**AAGCTT**GCCACCATGGGAAATGGTTCAGTG-3' containing the restriction site for HindIII and the reverse primer 5'-GGGCCC**GGTACC**GAAGTCCTTATCCCAGCC-3' containing the restriction site for KpnI were used to amplify the FL wt PKG II from RT brain cDNA.

b. 5' wt PKG II

The forward primer 5'-GCTAGC**AAGCTT**GCCACCATGGGAAATGGTTCAGTG-3'
containing the restriction site for HindIII and the reverse primer

5'-GGGCCC**GGTACC**GAGTTCATCAAATGTCCC-3' containing the KpnI restriction site
were used to amplify the regulatory domain of PKG II consisting of amino acids 1-397 of wt
PKG II using FL wt PKG II GFP-GW1 as a template.

c. FL PKG II G2A mut

To make a FL NM mut of PKG II the codon for glycine GGA was replaced with the codon
for Alanine G<u>C</u>A in the forward primer. The forward primer

5'-GCTAGC**AAGCTT**GCCACCATGG<u>C</u>AAATGGTTCAGTG-3' containing the restriction
site for HindIII and the reverse primer

5'-GGGCCC**GGTACC**GAAGTCCTTATCCCAGCC-3' containing the restriction site for
KpnI were used to amplify the FL PKG II G2A mut using FL wt PKG II GFP-GW1
construct as a template.

d. 5' PKG II G2A mut

To make a NM mut of PKG II regulatory domain the forward primer

5'-GCTAGC**AAGCTT**GCCACCATGG<u>C</u>AAATGGTTCAGTG-3' containing the restriction
site for HindIII and the reverse primer

5'-GGGCCC**GGTACC**GAGTTCATCAAATGTCCC-3' containing the KpnI restriction site were used to amplify the region encoding the amino acids 1-397 of PKG II using FL PKG II G2A mut GFP-GW1 as a template.

B. His-Cat PKG II

To amplify just the catalytic domain the primers 5'-AAACTT**AAGCTT**GCCGCCATGCATCACCATCACCATCACGCCACCCTGAACCGTGA-3' containing the restriction site HindIII and the reverse primer 5'-GGGCCC**GAATTC**GAGTTCATCAAATGTCCC-3' containing the EcoRI site were used to amplify the region encoding the last 366 amino acids of PKG II using FL PKG II as a template. The fragment was subcloned into the mammalian expression vector pCDNA3 (Invitrogen).

Figure 2: Constructs Generated for Transfecting mammalian cells.

All GFP constructs were subcloned in the mammalian expression vector GW1 and the His-tagged catalytic domain of PKG II was subcloned in the pCDNA3 vector (Invitrogen).
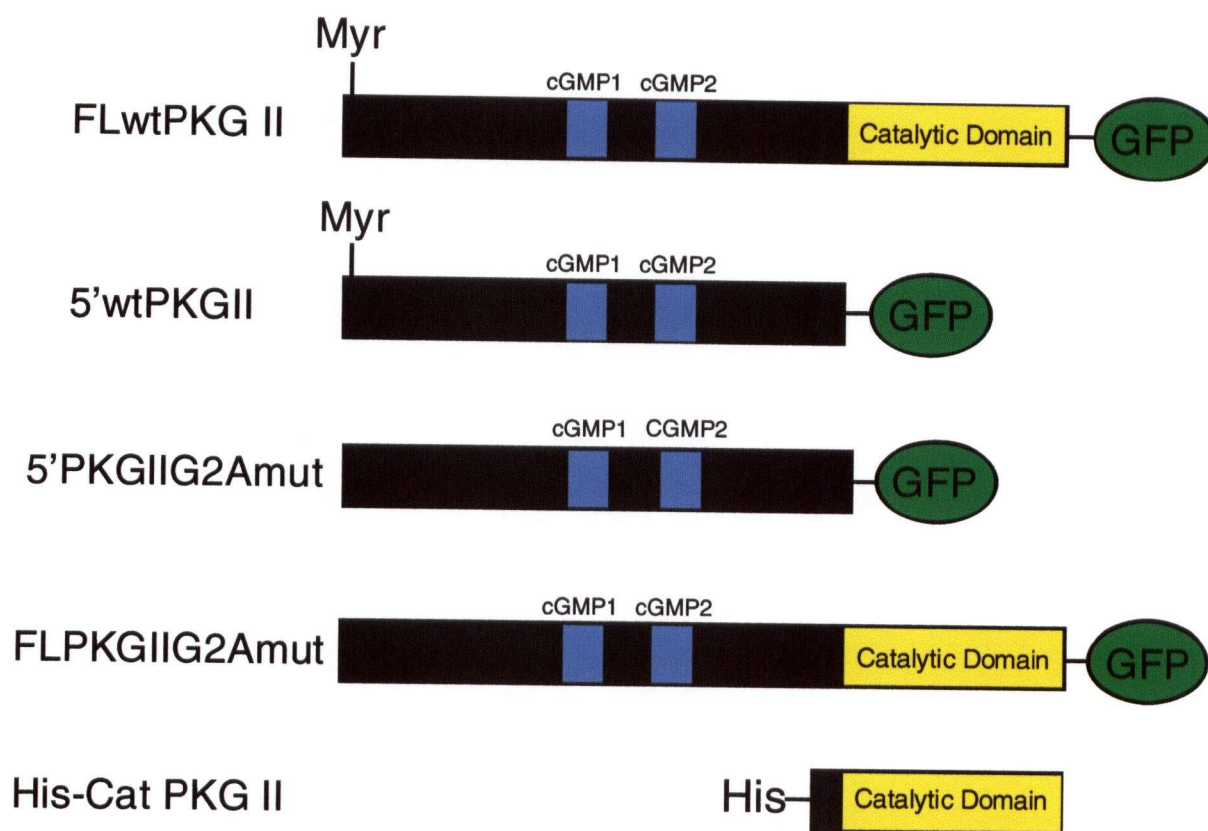
Figure 3: Western blot analysis of expression of various PKG II constructs in transiently transfected HEK-293 cells.

HEK-293 cells were lysed 24-48 hours post-transfection. Western blot was performed using rabbit GFP antibodies (See Table 2 for concentration used). Arrows indicate the bands corresponding to the expressed proteins.

42
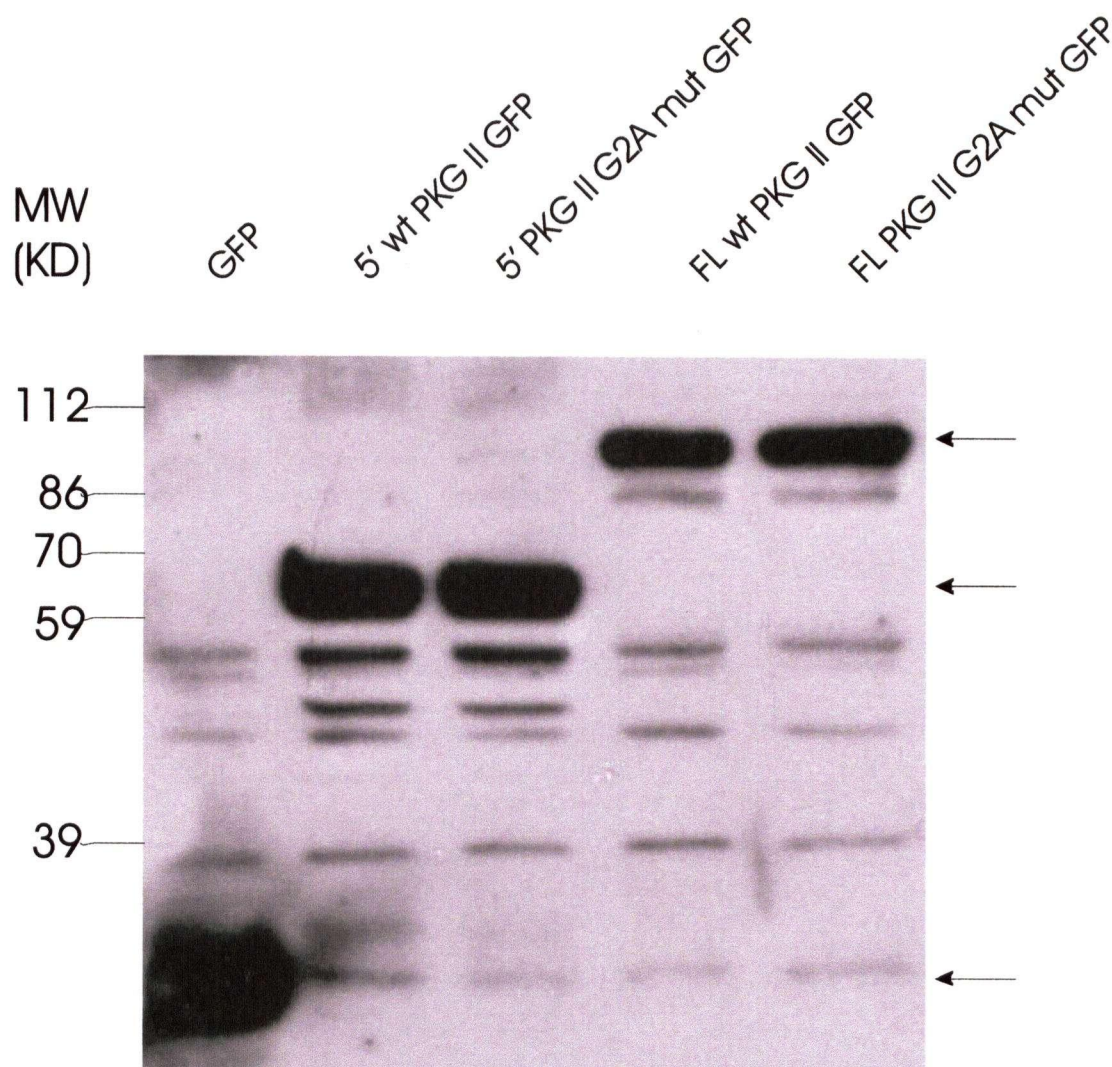
VIII. Subcellular Fractionation

Subcellular fractions were prepared from 6 whole brains of adult rats (young adults weighing 100-150 g) or 15 whole brains of rat pups (P0) as described earlier (Huttner et al., 1983) (Lin et al., 1998). All steps were performed on ice. Brains were homogenized with 9 strokes of a Dounce homogenizer at 900 rpm in 50 mL of homogenizing buffer (320 mM sucrose, 4 mM Hepes, pH 7.4, 1 mM EGTA, 1 mg/mL pepstatin and 200 mM PMSF). The lysate was centrifuged for 10 min at 1000 x g, which removed large debris and nuclei (P1). The snt (S1) was centrifuged at 12 000 x g to obtain the S2 fraction containing small cell fragments. The pellet was re-suspended in homogenizing buffer and centrifuged for 15 min at 13 000 x g, which resulted in a snt (S2') consisting of small compartments and a pellet of crude synaptosomal membranes. Homogenizing buffer was used to re-suspend the pellet. The sample was then homogenized at a very slow speed, followed by hypo-osmotic lysis by addition of 9 vol. of ice-cold water. This sample was centrifuged for 20 min at 33,000 x g to obtain heavy membranes (LP1) and a snt. The snt was centrifuged at 251,000 x g for 2 h, which yielded a snt of presynaptic cytosol (LS2) and a pellet (LP2) containing synaptic vesicles. The pellet was re-suspended in 40 mM sucrose. Protein conc. was determined using BCA protein assay kit. Fifty µg protein of each fraction were loaded on an SDS gel and were detected by western blotting.

IX. Cell Culture

1. Material

Product	Company	Cat. No.

HBSS	Invitrogen	14170-112
NBM	Invitrogen	21103-049
Papain	Sigma	P-3125
Trypsin Inhibitor	Sigma	P-9253
DNAse I	Sigma	DN-25
B-27 supplement	Invitrogen	17504-044
L-glutamine	Invitrogen	250300-81
DMEM	Invitrogen	121000-46
Penicillin/Streptomycin	Invitrogen	150700-63
Trypsin 0.25% EDTA	Invitrogen	252000-56
FBS, USA Origin	Invitrogen	261400-79
Poly-D-Lysine	Sigma	P-6407

2. Method

A. COS Cells and HEK-293 cells

Cells were obtained from American Type Culture Collection. Cells were maintained in an incubator containing 5% CO_2 at 37° C in DMEM supplemented with penicillin/streptomycin (100 U/mL) and 10% FBS. Cells were passaged every three to four days. For passaging cells, the medium was aspirated, washed once with pre-warmed (37° C) PBS and incubated in 1 mL trypsin (0.25% in PBS) for 2 min at 37° C. Ten mL of fresh PBS were added to cells and the solution was triturated with a 10-mL pipette a few times to detach cells. The solution with cells was centrifuged at 1000 x g for 3 min. The snt was aspirated and the cells re-suspended in 10 mL of pre-warmed fresh medium.

B. Neurons

Twenty four-well plates with cover slips were coated with poly-D-lysine (50 µg/mL) for 3 h. Hippocampal neurons were cultured as described previously (Brewer, 1995; Brewer et al.,

1993). Hippocampi and cortices were dissected from embryonic day 18-19 rat embryos and transferred to HBSS. Thalami were dissected from embryonic day 15 rat embryos. The tissues were digested in papain solution (containing 0.5mM EDTA and 1 mM CaCl2) by triturating with a 10 mL pipette gently a few times. The solution was incubated at 37° C for 10 min. The cells were spun down at 2500 rpm for 3 min. Cells were then re-suspended in trypsin inhibitor solution containing DNAse I and L-cysteine, and centrifuged for 3 min at 2500 rpm. The cell pellet was then re-suspended in 5 mL of complete NBM containing B-27 supplement and penicillin/streptomycin solution. Cells were counted using a haemocytometer and plated at a density of 100-150,000 per cover slip. The neurons were maintained in an incubator containing 5% CO_2 at 37° C. Fifty % of the medium was changed once every five days.

X. Cell Transfection

1. Material

Product	Company	Cat. No.
Lipofectamine 2000	Invitrogen	11668-027
Effectene	Qiagen	3014-25
OPTIMEM	Invitrogen	226000-50

2. Method

A. Lipofectamine 2000

Lipofectamine 2000 reagent was used to transfect COS cells and HEK-293 cells. Cells were passaged and plated in 6-well plates a day before transfection. DNA and Lipofectamine

reagent were mixed in 500 μL OPTIMEM at a ratio of 1:2.5 and incubated at room temp for 20 min. The medium was aspirated from the well and the mixture was added to the cells along with 1.5 mL of fresh complete DMEM. The cells were either lysed or fixed 24-48 h after transfection.

B. Effectene

Hippocampal neurons were transfected either on *div* 6 or *div* 10 with Effectene reagent. Two μg of DNA was used to transfect six wells in a 24-well plate. Two μg of DNA and 8 μL of enhancer solution were added to 150 μL of EC buffer. When two constructs were co-transfected, 1 μg each of the constructs was used. The mixture was incubated at room temp for 5 min and then 12-15 μL of Effectene reagent were added. The solution was gently vortexed for two seconds and incubated at room temperature for 10 min. One mL of fresh complete NBM was added to each tube containing the mixture of DNA and Effectene reagent. One hundred and forty μL of the mixture were added to each of the six wells containing 200 μL of complete NBM. The cells were incubated at 37° C and 5% CO_2 for 2.5 to 3 h after which the transfection mixture was aspirated and 1 mL of fresh complete NBM was added to each well. Cells were fixed 4-5 days after transfection.

XI. Immunocytochemistry

1. Material

Product	Company	Cat. No.
Fluoromount G	Electron Microscopy Sci.	17984-25

2. Method

All immunocytochemistry experiments were done on 24-well plates. One to two days after transfection COS cells and HEK-293 cells were fixed for 15 min with a solution containing 4% PFA and 0.1 M potassium phosphate. Cells were then washed three times with PBS containing 0.3% Triton-X (PBS-t). Cells were incubated for one h at room temp in 150-200 µL of the appropriate primary Ab in PBS containing 0.3% Triton-X (PBS-t) and 2% NGS (See table above for primary Ab conc). Cells were washed three times with PBS-t. Cells were then incubated with the appropriate fluorescent secondary Ab (see table above for secondary Ab conc) or biotinylated antibodies in case of DAB/ABC staining, for 1 h at room temp. Cells were washed 3 times with PBS-t and cover-slipped in Fluoromount G. Cells were stored in dark until analyzed. Hippocampal neurons were fixed in 100% methanol 4-5 days post transfection. Rest of the procedure was as described above.

For immunoperoxidase staining, the ABC method was used using Vectastain® ABC kit. Cells were incubated for 1 h at RT in PBS-t solution containing avidin-biotinylated horseradish peroxidase complex. Following a final series of rinses in PBS, the immunoreactivity was revealed using a nickel-enhanced DAB reaction.

For peptide inhibition studies 3 µg of PKG II Ab were incubated with 30 µg of peptide antigen (supplied by Affinity bioreagent, Inc) in one mL of PBS-t overnight at 4° C. Between 150 to 200 µL of the dilution was added to each well.

Table 1: Antibodies used in Immunocytochemistry

Primary Abs	Company	Dilution used	Host
GFP (AFP5002)	Qbiogene	1 in 1000	Mouse
GFP (132002)	Synaptic Systems	1 in 2000	Rabbit
PKG II Affinity purified	custom made	1 in 200	Rabbit
PKG I CT (KAP-PK005)	StressGen	0.25 μg/mL	Rabbit
PSD-95 (MA1-046)	ABR	1 in 200	Mouse
Synaptophysin (S5768)	Sigma	1 in 1000	Mouse
Synaptophysin (18-0130)	Zymed Laboratories, Inc.	1 in 1000	Rabbit

Secondary Abs	Company	Dilution used	Host
Alexa 488 anti-rabbit (A11034)	Molecular Probes	1 in 1000	
Fluorescein (FITC) Anti-Mouse IgG	Jackson ImmunoResearch	1 in 1000	Goat
Texas Red anti-rabbit IgG	Jackson ImmunoResearch	1 in 200	Donkey
Texas Red anti-mouse IgG	Jackson ImmunoResearch	1 in 200	Donkey

XII. Image Acquisition and Quantification

Fluorescence Images were acquired using Zeiss Axiophot microscope. Images were acquired using a 63X objective. At least fifteen cells in each category were randomly selected and analysed. Morphometric measurements were performed using Northern Eclipse image analysis software (Empix Imaging Inc.). Dendritic filopodia/spines (Hering and Sheng, 2001) and synaptophysin puncta were counted manually and logged into Microsoft Excel. One way ANOVA was performed to test the significance of the data.

XIII. Immunoprecipitation (IP)

1. Material

Product	Company	Cat. No.	
Protein A-Sepharose	Amersham	17-0780-01	

Primary Abs	Company	Dilution used	Host
PKG II Affinity purified	custom made	3 μg	Rabbit

2. Method

A. Cell Lysis

Cells were lysed in 500 μL of TEEN (50 mM Tris, pH7.4, 1 mM EDTA, 1 mM EGTA and 150 mM NaCl) containing 1 μg/mL peptatin A, 10 mM PMSF and 1 % Triton-X (unless indicated otherwise) for 20 min at 4° C with vigorous shaking. The cells were scraped and transferred to a 1.5 mL centrifuge tube and centrifuged at 10 000 rpm for 15 min. The snt was transferred to a fresh tube and used for experiment immediately or stored at -80° C.

Brains were homogenized with 9 strokes of a Dounce homogenizer at 900 rpm in 1 to 10 w/vol of the above mentioned lysis buffer that also contained 320 mM sucrose and 0.1% SDS. The homogenates were rotated for 20 min at 4° C for complete lysis to occur. The homogenates were then centrifuged for 20 min at 10 000 rpm and the snt transferred to fresh tube. The homogenates were either used immediately for experiments or stored at -80° C until further use.

B. Preparation of Protein A-Sepharose beads (CLA4) for IP.

Two mL of beads slurry were suspended in 10 mL of 50 mM Tris, pH 7.4, by rotating for 30 min. at 4° C. Beads were collected by centrifugation at 1000 x g for 5 min. Two mg/mL of crystallized BSA was added to the beads and the beads were re-suspended in 10 mL of 50 mM Tris, pH 7.4, by rotating for 10 min at 4° C. The process was repeated twice. After the last centrifugation step, the Tris solution was aspirated and 1 vol. of TEEN + 1% Triton-X was added to the beads. The beads were stored at 4° C until further use.

C. IP procedure

All steps were done at 4° C. For the IP experiments 500 µL of cell lysate was pre-cleared with 20 µL of protein A-Sepharose slurry. Samples were centrifuged at 5000 rpm for 2 min and the snt transferred to a fresh tube. The snt was incubated with primary Ab (see table above for conc of Ab used) overnight. Eighty µL of bead slurry were added the next day and the samples rotated for 1 h. Beads were collected by centrifugation at 5000 rpm for 2 min. The beads were washed three times with the lysis buffer mentioned under cell lysis for two

min each. Beads were collected between washes by centrifugation at 5000 rpm for 2 min.

The beads collected after the final wash were re-suspended in 50 μL of SDS-sample loading

buffer and boiled for three min to extract immunoprecipitated proteins.

XIV. Western Blotting

1. Material

Product	Company	Cat. No.
ECL Western Blotting reagent	Amersham	RPN2106
Kodak BioMax mR-1 Film	Kodak/Amersham	V8701302
Hybond-C Nitrocellulose memb.	Amersham	RPN303C
Protein A-Sepharose	Amersham	17-0780-01

2. Method

Ten % polyacrylamide gels were prepared as described in a manual from BioRad. PAGE was

performed at constant 30 mamp in the stacking gel and 36 mamp in the separating gel.

Proteins were transferred to nitrocellulose membranes using the wet transfer method and the

Bio Rad transfer system. After transfer, membranes were washed twice with TBS-t (25 mM

Tris, 0.8% NaCl, 0.02% KCl, pH 7.6 and 0.05% TWEEN-20) for 10 min each. They were

then incubated in blocking solution (6% milk in TBS-t) for 2 h at room temperature.

Membranes were the rinsed three times with TBS-t and incubated with the specific primary

Ab overnight at 4° C in TBS-t containing 1% BSA and 0.02% Na Azide. The membranes

were washed three times for 10 min each and incubated with species specific horse-radish-

peroxidase-linked secondary Abs for 1 h at room temp in TBS-t containing 1% BSA and

0.02% Na Azide. Membranes were again washed three times for 10 min each at room temperature. Membranes were finally incubated for two min at room temperature in ECL reagents and exposed to X-ray films in a dark room to visualize protein bands.

Table 2: Antibodies used in Western Blotting

Primary Abs (Cat. No.)	Company	Dilution used	Host
Actin (A 2066)	Sigma	1 in 500	Rabbit
GFP (83621-1)	Clontech	1 in 1000	Mouse
NR-1 (114011)	Synaptic Systems	1 in 10,000	Mouse
PKG II Affinity purified	custom made	1 in 200	Rabbit
PKG I CT (KAP-PK005)	StressGen	0.25 μg/mL	Rabbit
PSD-95 (MA1-046)	ABR	1 in 2000	Mouse
Synaptophysin (S5768)	Sigma	1 in 400	Mouse
VASP (ALX-804-177)	Alexis Biochemicals	1 in 1000	Mouse
Phospho S239 VASP (ALX-804-240)	Alexis Biochemicals	1 in 1000	Mouse

Secondary Abs	Company	Dilution used	Host
Anti-Rabbit HRP (NA934V)	Amersham	1 in 5000	Donkey
Anti-Mouse HRP (NA931V)	Amersham	1 in 5000	Sheep

Results

I. Generation and characterization of PKG II antibodies

To characterize the distribution of PKG II protein in the brain and to study the subcellular distribution of PKG II, we raised a polyclonal antibody in rabbit against a 14 amino acid peptide corresponding to rat PKG II sequence. The sequence is in the hinge region of PKG II. The hinge region was chosen as it exhibits the highest sequence divergence from PKG I. The sequence was also put through the BLAST program on the NCBI web site to make sure there were no other similar sequences in the database. The exsanguination bleed was initially tested and affinity purified using sulpholink® columns (see Materials and Methods). The affinity purified antibody (PKG II antibody) was used for further studies.

1. PKG II antibody detects FLwt PKG II but not 5'wt PKG II in HEK-293 cells.

To test the specificity of PKG II antibody, we used transiently expressed FL PKG II, which contains the antibody recognition site, and the regulatory domain of PKG II (5'wt PKG II) in HEK-293 cells. For this study the FL PKG II tagged to GFP and 5'wt PKG II tagged to GFP were subcloned in the GW-1 vector and transfected into HEK-293 cells as described in Materials and Methods. HEK-293 cells were used because of their high transfection efficiency. On the one hand, as shown in Fig. 4, the PKG II antibody detected only the FL PKG II GFP (~98 kD) and not the 5'wt PKG II (~65 kD). On the other hand, a rabbit polyclonal GFP antibody detected both the bands as they are both tagged with GFP (See

Table 2 for antibody concentrations). The PKG II antibody also detected a smaller band in Lane Two on the right panel in Fig. 4 (n=5). It is possible that this band corresponded to a cleaved form of PKG II. Previous studies on rat intestine have shown that the 86 kD PKG II is cleaved to a protein of ~72 kD (de Jonge, 1981; Jarchau et al., 1994) and the lower band shown in Fig. 4 corresponded to the size of ~72 kD PKG II.

These results show that PKG II antibody recognized transfected FL wt PKG II. The results also indicate that HEK-293 cells probably do not express endogenous PKG II.

Figure 4: Affinity purified antibody detects recombinant PKG II expressed in HEK-293 cells.

Cells overexpressing GFP tagged regulatory domain (5' wt PKG II GFP) and FL PKG II (FL wt PKG II GFP) were lysed and Western blot anaylysis was performed using rabbit polyclonal GFP antibodies (left panel) and affinity purified antibody against PKG II (right panel). PKG II antibody recognizes only the FL wt PKG II and not 5' wt PKG II, which does not have the antibody recognition site. In contrast, the GFP antibody detected both the recombinant proteins (n=3).
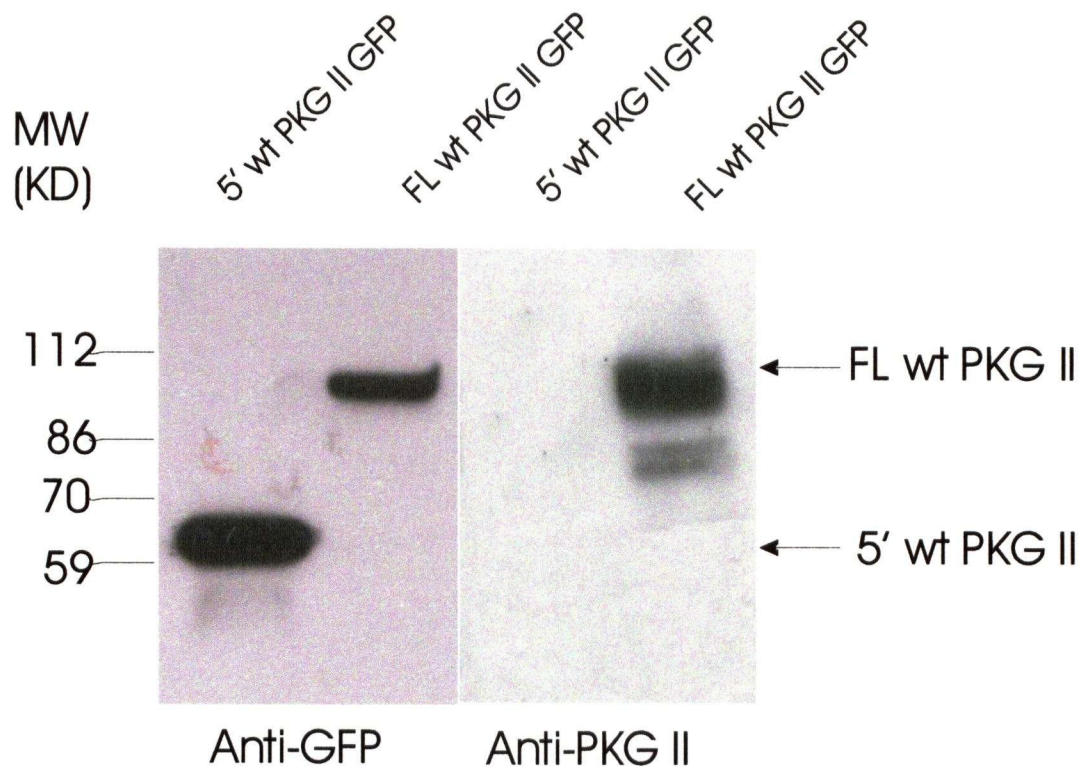
2. Immunocytochemical detection of His-tagged PKG II catalytic domain (His-PKG II cat) protein in HEK-293 cells transfected with His-PKG II cat cDNA using affinity purified PKG II antibody.

Next, we wanted to check if the PKG II antibodies can be used to immunocytochemically detect recombinant PKG II expressed in HEK cells. HEK cells were transfected with a His-tagged PKG II catalytic domain and immunocytochemistry was performed using the PKG II antibodies and DAB/ABC method. In this study we used just the catalytic domain, containing the PKG II antibody recognition site, to find out if the PKG II antibody can detect not only the full length, but also part of the PKG II molecule. The catalytic domain was tagged with hexa histidine to confirm the expression of the protein by a second method. PKG II antibodies stained HEK cells transfected with His-PKG II cat cDNA but not the vector alone (Fig. 5A; n=3). Stained cells showed cytoplasmic localization of the PKG II catalytic domain, although the catalytic domain of PKG II appeared to be slightly more concentrated in the perinuclear area. Western blotting was performed using histidine antibodies, and as shown in Fig 5C, a protein corresponding to the His-cat PKG II is detected only in lysates overexpressing the His-cat PKG II. These results demonstrate that the PKG II antibody can recognize recombinant PKG II both by Western blot and immunocytochemical methods.

The results demonstrate that PKG II antibody can be used for immunocytochemistry.

Figure 5: Immunodetection of recombinant PKG II protein in HEK-293 cells transiently transfected with PKG II catalytic domain cDNA using DAB/ABC method.

His-tagged PKG II catalytic domain expressing the last 366 amino acids of PKG II was transfected into HEK cells using lipofectamine reagent. 24 well plates were transfected with the vector alone (A) or with His-PKG II cat cDNA (Cell in B). Cells were stained using the PKG II antibody. As shown, staining was detected only in cells transfected with His-PKG II cat cDNA, but not the vector. Histidine antibodies were used to detect His-PKG II cat in lysates from HEK cells transfected with His-Cat PKG II (Fig. 5C right panel) and vector alone (Fig 5C left panel). There were no major bands detected in lysates from cells transfected with the vector alone (n=3).
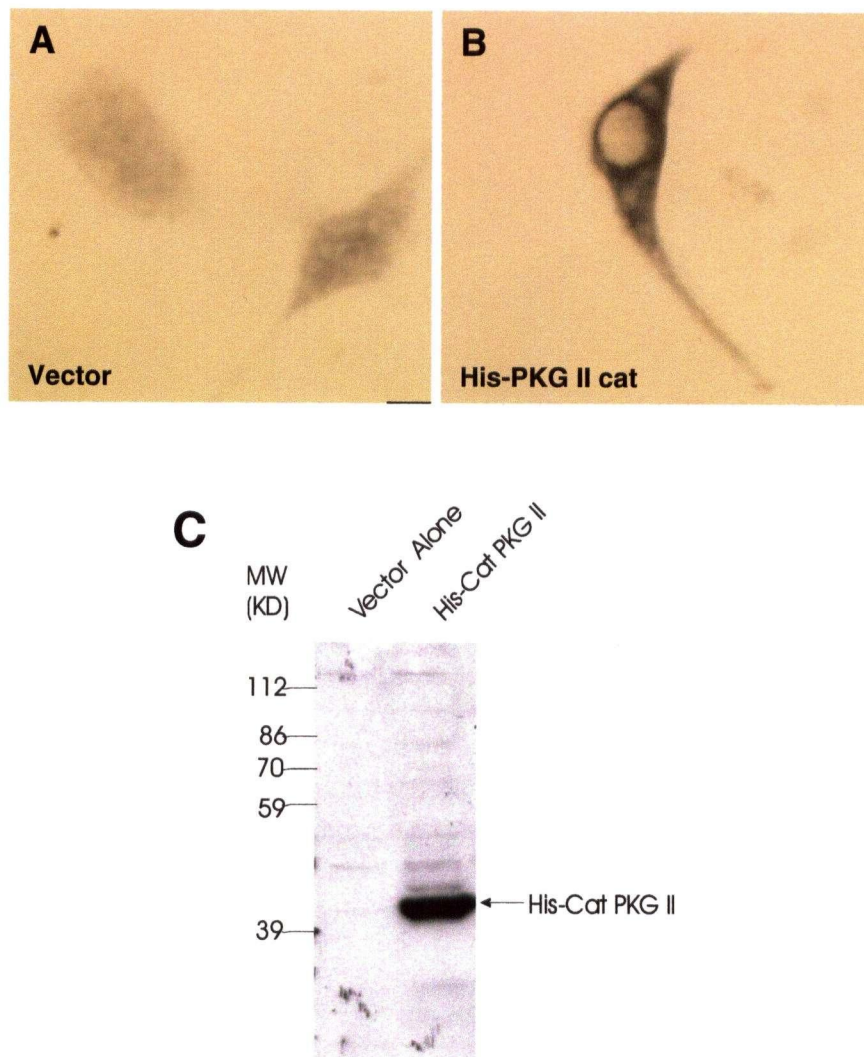
3. PKG II antibody detects a major band corresponding to PKG II in crude lysates from various brain regions.

Although PKG II antibody recognized recombinant PKG II expressed in HEK-293 cells, we wanted to verify that PKG II antibody could also detect endogenous PKG II from brain lysates. PKG II antibody detected a major band ~86 kD corresponding to the size of PKG II in lysates from various brain regions. The band was detected in all regions tested except cerebellum (Fig. 6, n=3). PKG II expression appeared to be highest in cortex and striatum. PKG II was also detected in hippocampus, hypothalamus and thalamus. The 86 kD band was conspicuously absent from cerebellar lysate. These results corelated with the distribution of PKG II mRNA shown by situ hybridization by El-Husseini et.al. (el-Husseini et al., 1995). Actin antibodies were used on the same membrane to confirm equal amounts of protein were loaded in each lane.

In order to compare the distribution of PKG II with PKG I, Western blotting was performed using PKG I antibodies on the same lysates. In contrast to PKG II, PKG I showed the highest expression in cerebellum. PKG I was also present in hypothalamus and weakly expressed in hippocampus and cortex. It was almost absent in thalamus and striatum. These data also agreed with previous studies on PKG I distribution, where it was shown to be expressed in the Purkinje cells of the cerebellum, the dorsomedial hypothalamus and hippocampus (Arancio et al., 2001; El-Husseini et al., 1999; Kleppisch et al., 1999; Lohmann et al., 1981).

These results indicate that the PKG II antibody recognized endogenous PKG II in brain lysates and PKG II was widely distributed in the brain.

Figure 6: Western blot analysis of PKG II and PKG I expression in various brain regions.

Affinity purified PKG II antibody and a commercially available rabbit polyclonal PKG I antibody were used to detect the expression of PKG II and PKG I in various brain regions. PKG II is present in all the brain regions examined except cerebellum, where it is conspicuously absent. PKG II has the highest expression in Cortex, but is also highly expressed in striatum, hippocampus and thalamus. In contrast, PKG I had the highest expression in cerebellum followed by hypothalamus and weakly expressed in hippocampus and cortex and almost absent in striatum and thalamus. Actin antibodies were used to confirm equal amounts of protein were loaded in each well (n=3).
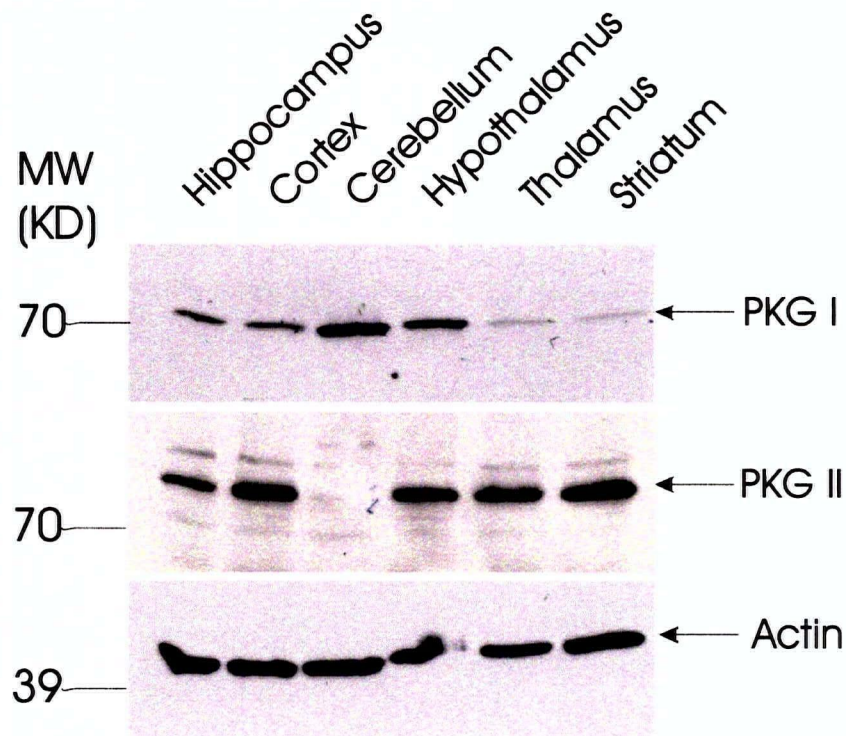
4. PKG II antibody immunoprecipitates a ~86 kD band from thalamic lysates and lysates of hippocampal neurons in culture.

We also wanted to test if the PKG II antibody could be used for immunoprecipitation. Thalamic lysates were used as there was high level of PKG II but no detectable PKG I protein. Lysates from hippocampal neurons in culture were also used to find out if there was any PKG II protein so that the hippocampal neurons can be used as a model to study PKG II function. The PKG II antibody immunoprecipitated a ~86 kD band from thalamic lysates (Fig. 7A) and from lysates of 10 *div* and 15 *div* hippocampal neurons in culture (Fig. 7B). As a negative control PKG I antibody was used and it did not immunoprecipitate any proteins from thalamic lysates (Fig. 7A lane 1). When primary antibodies were omitted as a negative control in the immunoprecipitation experiment using lysates from hippocampal neurons in culture, no proteins were detected (Fig. 7B lane 1). PKG II antibody also detected a protein band corresponding to the size of PKG II from crude extracts of both 10 *div* and 15 *div* hippocampal neurons in culture.

These results indicate that PKG II was present in hippocampal neurons in culture and that the PKG II antibody could be used for immunoprecipitation studies.

Figure 7: Immunoprecipitation of PKG II from rat thalamic lysates and lysates of hippocampal neurons in culture.

Affinity purified PKG II antibody was used for immunoprecipitation of PKG II from lysates of rat thalami (A) and 10 *div* and 15 *div* hippocampal neurons in culture (B). A single ~86 kD band was immunoprecipitated. The band was also detected in the extracts. As a negative control, PKG I antibody (A left lane) was used or primary antibodies were omitted (B lane far left) (n=2).
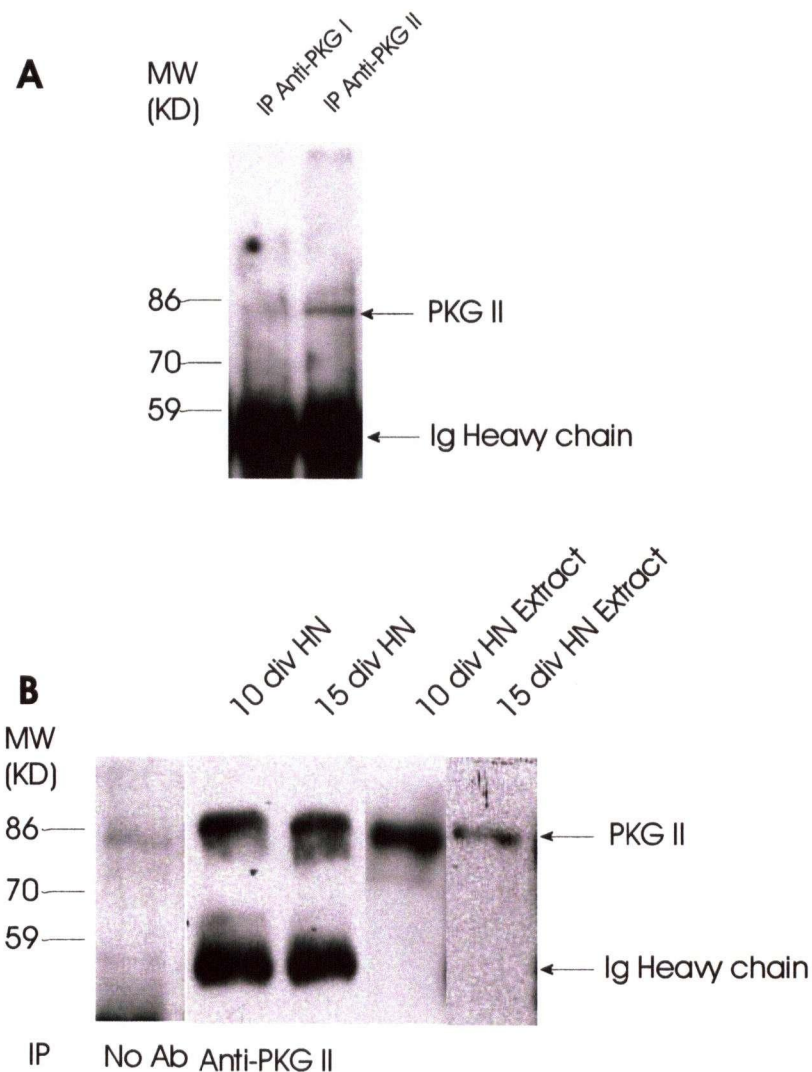
5. Preincubation of PKG II antibody with the antigenic peptide abolishes staining in hippocampal neurons in culture.

PKG II antibody appeared to work well in Western blotting applications, but we wanted to confirm the endogenous PKG II expression in hippocampal neurons in culture using immunocytochemistry. We also wanted to see if PKG II antibody staining could be inhibited by the peptide against which it was raised. PKG II antibody was used to stain 15 *div* hippocampal neurons in culture. Neurons were double stained with PKG II antibody and an antibody against the presynaptic marker protein, synaptophysin (Fig. 8A). Texas red conjugated mouse IgG and FITC conjugated rabbit IgG secondary antibodies were used to detect the staining (See Table 1 for Ab concentrations). PKG II antibody stained both the cell body and processes. To test the specificity of the PKG II antibody, the antibody was preadsorbed with the peptide against which the antibody was raised (see Materials and Methods for detailed description of methods). The PKG II antibody peptide solution also containing synaptophysin antibody was used to stain hippocampal neurons in culture. As shown in Figure 8B, preadsorption of PKG II antibody with the peptide abolished the staining of PKG II, but left the synaptophysin staining intact. This indicates that the peptide specifically bound to PKG II antibody.

These results indicate that the PKG II antibody specifically recognized PKG II.

Figure 8: Immunodetection of PKG II in hippocampal neurons in culture and preadsorption of PKG II antibodies with the antigenic peptide.

Affinity purified PKG II antibodies were used to detect endogenous PKG II (A) in 15 *div* hippocampal neurons in culture. As a negative control PKG II antibodies were preadsorbed with the peptide (B) against which it was generated and that completely abolished the staining. Synaptophysin antibodies were also used to make sure that the peptide was specific in abolishing the staining by PKG II antibody and not synaptophysin antibody. PKG II antibody preincubated with antigenic peptide (Ab+peptide) (n=3).
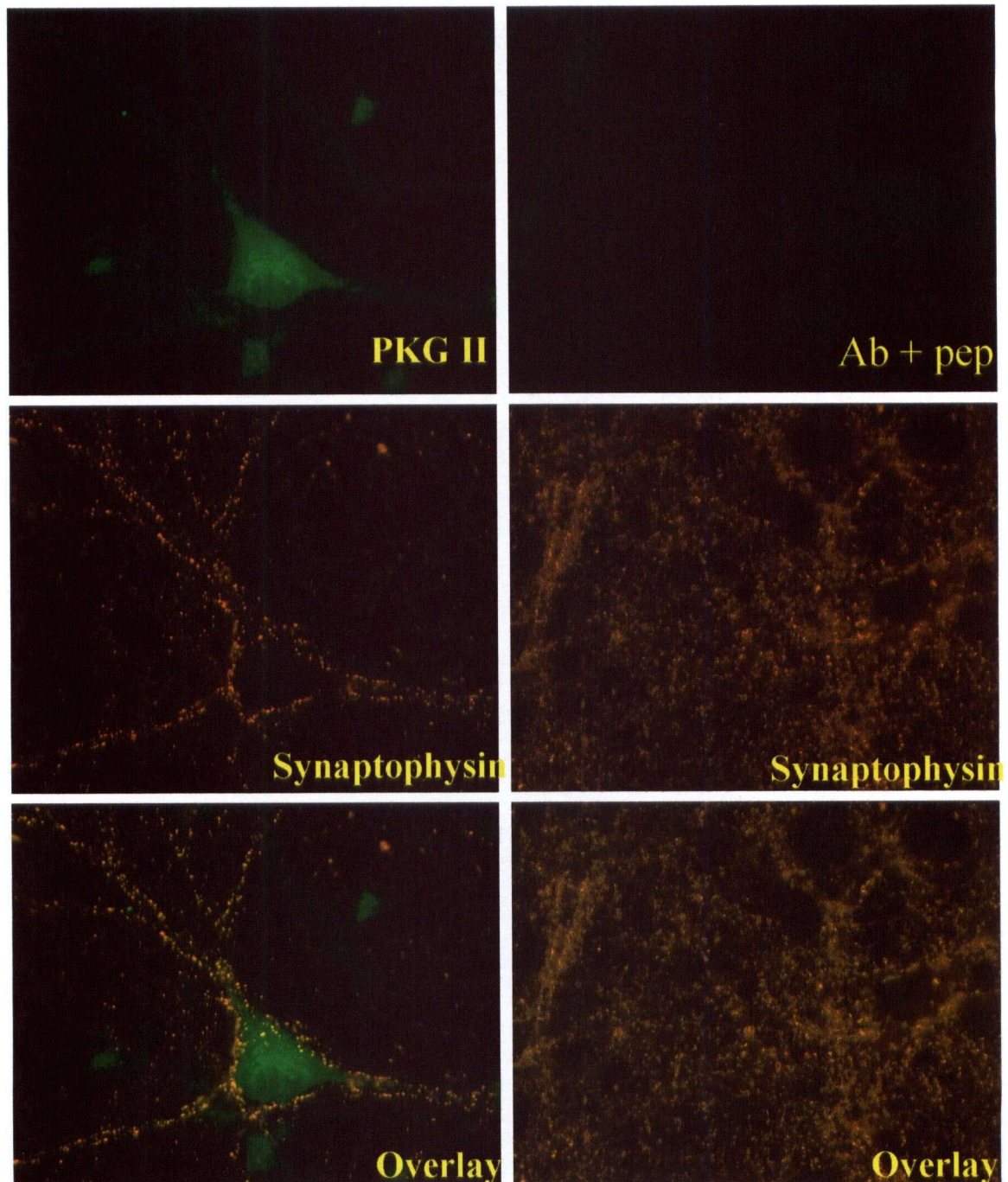
6. Immunodetection of PKG II in thalamic and cortical neurons in culture.

As PKG II appeared to be present in the cortical and thalamic lysates, we wanted to check if the PKG II antibody detected PKG II in thalamic and cortical neurons in culture. PKG II antibody was used to detect PKG II in thalamic and cortical neurons in culture. Texas red conjugated mouse IgG and FITC conjugated rabbit IgG secondary antibodies were used to detect the staining. As shown in Fig. 7 PKG II appeared to be present in the cell bodies of both thalamic (9A) and cortical (9B) neurons in culture. PKG II was also present in the dendritic processes of thalamic neurons and partially co-localized with the presynaptic marker when double stained with synaptophysin antibody (Fig. 9C). PKG II also had a punctate distribution in the thalamic neuron processes suggestive of membrane targeting (Fig. 9C green).

Figure 9: Immunodetection of PKG II in thalamic and cortical neurons in culture.

Affinity purified antibodies also detected PKG II in thalamic (A) and cortical (B) neurons in culture. It was present in both the cell body (A) and processes (C) of thalamic neurons in culture and appeared to partially co-localize (arrow heads in C) with the presynaptic marker synaptophysin (n=2).
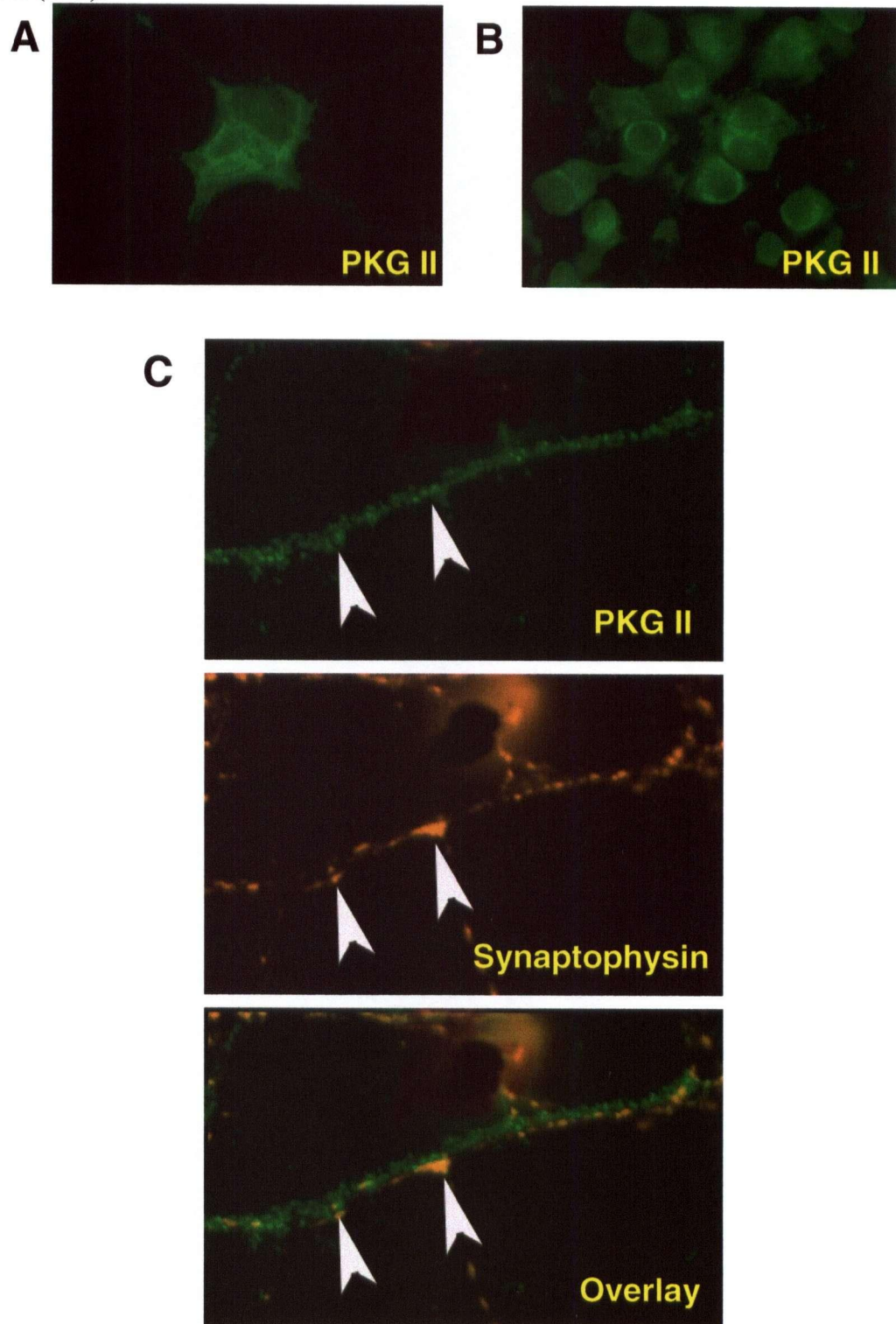
II. Subcellular distribution of PKG II in the brain and hippocampal neurons in culture.

Although the general tissue distribution of PKG II is known, its subcellular distribution in neurons is poorly described. Numerous synaptic functions have been attributed to cGMP and PKG (see Introduction). However, there is no evidence for the presence of PKG II in the synapse so far. Wang and Robinson found at least three proteins in the peripheral membrane fractions from synaptosomes that were phosphorylated in a PKG specific manner (Wang and Robinson, 1995) and they suggested that PKG is likely to be present in the synapse. Hence we chose to investigate the subcellular distribution of PKG II in the brain and hippocampal neurons in culture to see if PKG II was targeted to the synapse.

1. PKG II is present in both the pre and postsynaptic fractions of adult rat brain subcellular fractions.

Western blot analysis of adult rat brain subcellular fractions revealed that PKG II is predominantly expressed in the synaptic vesicle fraction (LP2) and small compartments (S2') containing small vesicles other than the vesicles in nerve endings (Fig. 10) (Huttner et al., 1983). PKG II was also present in the synaptosomal membrane fraction (LP1). PKG II appeared to be predominantly associated with membrane fractions and had a subcellular fractionation profile that partly overlaped with NR1, which is enriched in the postsynaptic density (PSD). PKG II was conspicuously absent from the presynaptic cytosol (LS2), again suggestive of membrane association. In fact PKG II has been shown to be membrane associated in intestinal brush borders and in HEK-293 cells (Jarchau et al., 1994).

To compare the subcellular distribution of PKG I with PKG II, we used PKG I antibodies on the same membranes. In contrast to PKG II, PKG I was very weakly expressed in all the synaptic fractions tested.

The subcellular fractions were also immunoblotted with the synaptic markers NR1 (Ehlers et al., 1995) and synaptophysin to make sure that the fractions were clean and not contaminated with proteins belonging to a different fraction. Actin antibodies were used to confirm equal amounts of protein were loaded in the wells.

These results indicated a membrane association and synaptic localization for PKG II. They also showed that PKG I and PKG II were enriched in different fractions leading to the hypothesis that most of the synaptic functions attributed to cGMP are likely to be mediated by PKG II and not PKG I.

Figure 10: Subcellular fractionation of PKG II in adult rat brain.

Homogenate (H), small cell fragments (S2), small compartments (S2'), synaptosomal membrane fraction (LP1), presynaptic cytosol (LS2), synaptic vesicles (LP2). Subcellular fractions were prepared from 6 adult rat brains. PKG II appeared to be in the LP1 and LP2 fraction indicating a synaptic localization. In contrast PKG I was very weakly expressed in both the LP1 and LP2 fractions. Antibodies against NR1 and synaptophysin were used to make sure the fractions were not contaminated. Actin antibody was used to confirm equal amounts of proteins were loaded in each lane (n=3).
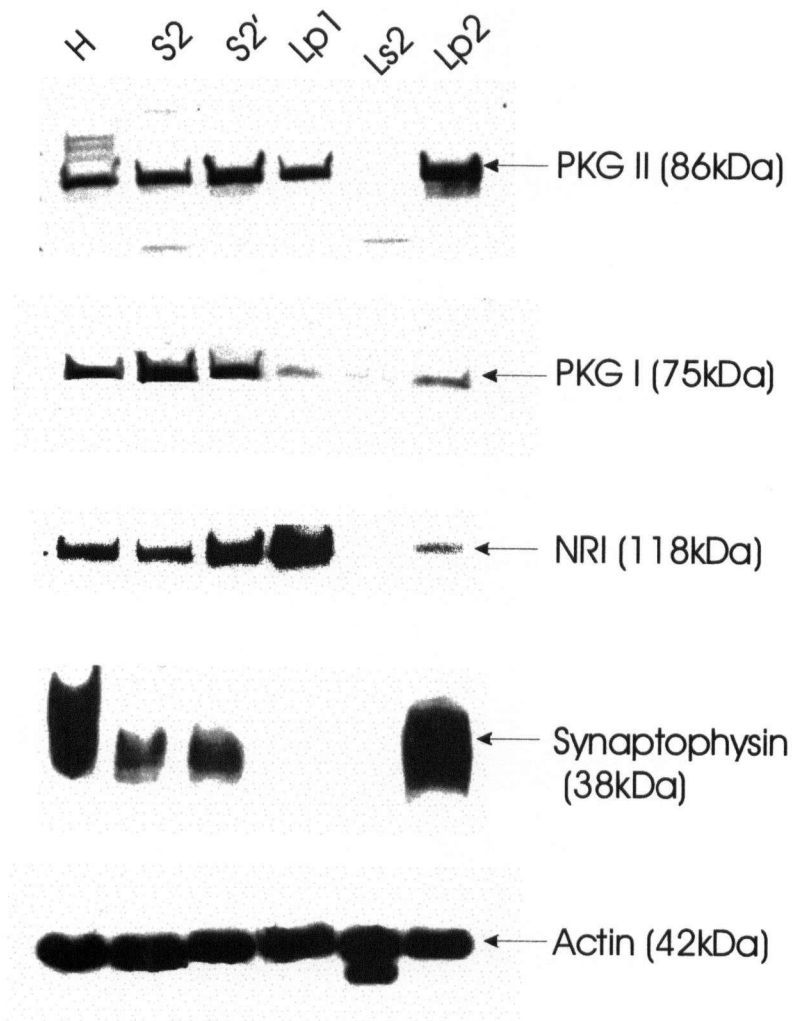
3. PKG II is predominant in the synaptosomal membrane fraction in embryonic rat brain subcellular fractions.

Western blot analysis of embryonic rat brain subcellular fractions revealed that PKG II was enriched in the synaptosomal membrane fraction (LP1) (Fig. 11). It appeared to be absent from the large debris and nuclei fraction (P1) and small cell fragments fraction (S2). PKG II had a profile similar to PSD-95, which is highly enriched in the PSD.

PKG I by contrast was very weakly expressed in the synaptosomal membrane fraction (LP1), and was enriched in small cell fragments fraction (S2) just like in adult subcellular fractions.

Figure 11: Subcellular fractionation of rat embryonic brains.

Whole brain homogenate (H), large debris and nuclei (P1), small cell fragments (S2), synaptosomal membrane fraction (LP1). Subcellular fractions were prepared from 15 P0 rat pups. PKG II expression seems to be highest in the LP1 fraction and has a profile similar to that of PSD-95, a postsynaptic density enriched protein. PSD-95 antibody was used to make sure the fractions were clean. The membrane was blotted with actin antibody to make sure equal amounts of protein were loaded in each lane. The result shown is a representative of results from three different experiments (n=3).
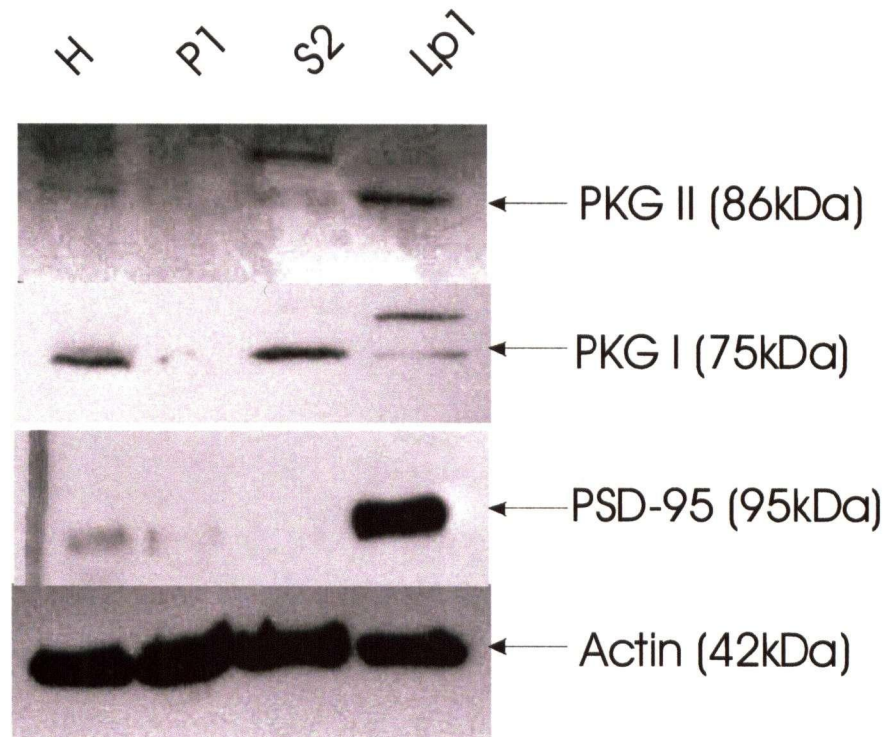
4. PKG II partially colocalizes with the presynaptic marker synaptophysin.

We wanted to test if endogenous PKG II was targeted to synaptic sites in hippocampal neurons in culture. Hippocampal neurons were double labeled for PKG II and the presynaptic molecular marker synaptophysin (Fig. 12a top, middle and bottom). Texas red conjugated mouse IgG and FITC conjugated rabbit IgG secondary antibodies were used to detect the staining. PKG II stained both the cell body (Fig. 8A) and dendritic processes (Fig. 12b top panel) in hippocamapal neurons. PKG II was present in the perinuclear area in the cell body (Fig. 8A top panel). The PKG II and synaptophysin merged pictures (Fig. 12a, b and c bottom panels) showed that PKG II and synaptophysin partially co-localize in hippocampal neurons. A magnified portion of Fig. 12b is shown in Fig. 12c. At higher magnification (Fig. 12c) a slight shift in fluorescence is observed, which was indicative of the proteins located at opposite sides of the synapse. As synaptophysin is a presynaptic marker, PKG II appeared to be on the postsynaptic side.

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Figure 12: Immunocytochemical localization of PKG II and the presynaptic marker synaptophysin in hippocampal neurons in culture.

Endogenous PKG II and synaptophysin were double labeled using affinity purified rabbit PKG II antibody (a top) and mouse synaptophysin antibody (a middle) and merged (a bottom). The right panel [b top (PKG II, b middle (synaptophysin) and b bottom (merged)] shows a magnified process. A further magnification of a portion of the process is shown in c top (PKG II), c middle (synaptophysin) and c bottom (merged), (n=4).
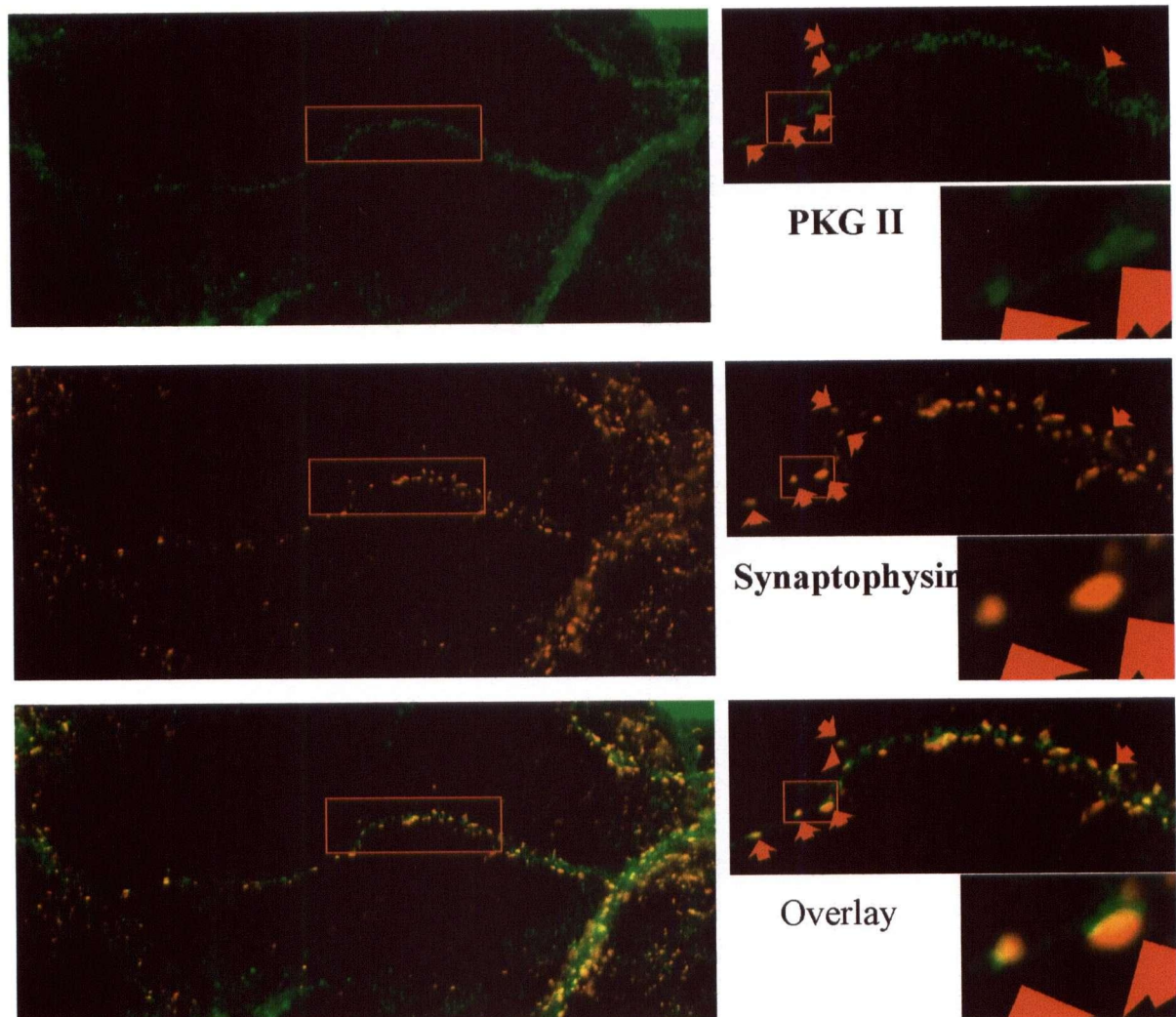
5. PKG II partially co-localizes with the postsynaptic molecular marker PSD-95.

Based on the results obtained from the study above, we wanted to test if PKG II co-localized with the postsynaptic marker PSD-95 in hippocampal neurons in culture. Hippocampal neurons were double labeled with PKG II and the postsynaptic molecular marker PSD-95 antibodies (Fig. 13a top, middle and bottom). Texas red conjugated mouse IgG and FITC conjugated rabbit IgG secondary antibodies were used to detect the staining. The PKG II and PSD-95 merged pictures (Fig. 13a and 13b bottom panels) shows that PKG II and PSD-95 partially co-localize in hippocampal neurons. At higher magnification (13b bottom panel) no shift in fluorescence was observed, which indicated that the proteins are localized in the same compartment. This indicates that PKG II targets to the postsynaptic side in hippocampal neurons in culture.

These results show that endogenous PKG II was targeted to synaptic sites in hippocampal neurons in culture.

Figure 13: Immunocytochemical localization of PKG II and the postsynaptic marker PSD-95 in hippocampal neurons in culture.

Endogenous PKG II and PSD-95 were double labeled using affinity purified rabbit PKG II antibody (a top) and mouse PSD-95 antibody (a mid.) and merged (a bottom). The right panel [b top (PKG II), middle (PSD-95), bottom (merged)] shows a magnifed process (n=4).

II. N-Myristoylation is important for proper targeting of PKG II in COS-1 cells, HEK-293
cells and hippocampal neurons in culture.

de Jonge first showed that PKG II activity is mainly present in the particulate fraction in the

intestinal brush borders (de Jonge, 1981). On the one hand, N-myr has been shown to be

important for targeting of rat PKG II to membranes in COS-1 cells and intestinal epithelial

cells (Vaandrager et al., 1996; Vaandrager et al., 1998). The mouse PKG II isoform, on the

other hand, when expressed in COS-1 cells appeared to be localized mostly (98%) in the

cytosol (Uhler, 1993). However, all of these localization data were based on measurements

of kinase activity and Western blotting rather than immunocytochemistry. We constructed

various PKG II GFP fusion proteins including FL PKG II G2A mutant, which has been

shown to be non-myristoylated (Vaandrager et al., 1996), 5' PKG II G2A mutant (regulatory

domain of PKG II that is non-myristoylated) and 5'wt PKG II (regulatory domain of wt PKG

II) (See Materials and Methods for details on generation of constructs) to test the importance

of N-myr in targeting of PKG II in COS-1 cells, HEK-293 cells and hippocampal neurons in

culture.

1. N-myr is important for targeting of PKG II to membranes and probable Golgi compartments in COS-1 cells.

COS-1 cells were transfected with FLwt PKG II and FL PKG II G2A mutant using

lipofectamine reagent. Cells were fixed 24 h post transfection with 2% PFA and stained with

rabbit GFP antibody and Alexa Fluor 488 anti-rabbit IgG secondary antibody to visualize the

recombinant proteins. As shown in Fig. 14A left panel FLwt PKG II was localized

predominantly in the perinuclear region in what appeared to be Golgi apparatus. FLwt PKG

II also localizes to the membrane and tips of filopodia like structures. By contrast, FL PKG II G2A mutant was diffuse (Fig. 14A right panel) and did not show any membrane localization or concentration in the perinuclear region, thus confirming that N-myr is important for membrane localization of PKG II in COS-1 cells.

We did not observe nuclear localization of the recombinant PKG II proteins in COS cells.

Figure 14: Immunocytochemical localization of FLwt PKG II GFP and FL PKG II G2A mutant GFP in COS-1 cells.

FLwt PKG II and FL PKG II G2A mutant were transfected into COS-1 cells with lipofectamine reagent. Cells were fixed with 2% PFA and stained with rabbit GFP antibodies and corresponding fluorescent secondary antibodies. Expressions of FLwt PKG II GFP (A) and FL PKG II G2A mutant (B) in COS-1 cells (A) are shown. Arrows in A indicate membranous staining and arrowheads show concentration of the protein in tips of filopodia like structures. At least 20 cells from each transfection category from two different batches of transfection were analysed.
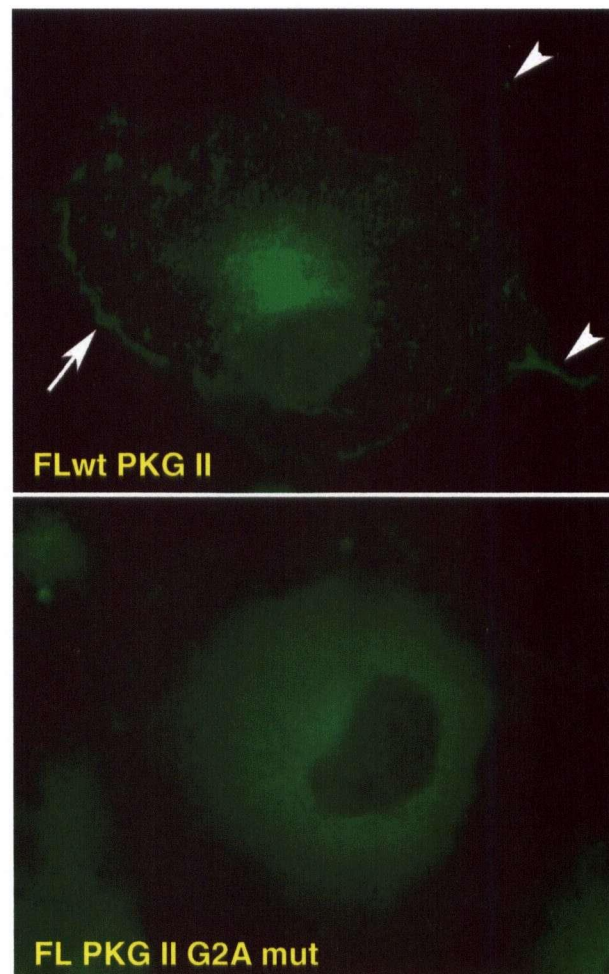
2. N-myr is important for targeting of PKG II to membranes and probable focal adhesion points in HEK-293 cells.

We also wanted to test the localization of the various PKG II recombinant proteins in HEK-293 cells in order to gain more insight into the functions of different structural regions of PKG II. 5'wt PKG II GFP (a), 5'PKG II G2A (b), FLwt PKG II GFP (c), FL PKG II G2A mutant (d) and GFP expressing vector alone (e), were all transiently transfected into HEK-293 cells using lipofectamine reagent. Cells were fixed 24 hours post transfection using 2% PFA and stained with rabbit GFP antibody and Alexa Fluor 488 anti-rabbit IgG secondary antibody to visualize the recombinant proteins. Both 5'wt PKG II and FLwt PKG II were concentrated in the perinuclear area, just as observed for FLwt PKG II in COS-1 cells, and also appeared to be targeted to the membrane (Fig. 15 a&e). The myristoylated forms are also concentrated at the distal ends of filopodia like structures. What was also striking was that the 5'wt PKG II transfected cells (Fig. 15 b) appeared to have more filopodia like structures than the FLwt PKG II (Fig. 14 e) or the non-myristoylated PKG II proteins (Fig. 15 c,d, and f) and also appeared to be localized in probable focal adhesion points (Fig. 15b). The non-myristoylated forms [5' PKG II G2A mutant (Fig. 15 c&d) and FL PKG II G2A mutant (Fig. 15 f)] and GFP (Fig. 15 g) had a much more diffuse localization. As in COS-1 cells, we did not observe any nuclear translocation of any of the PKG II recombinant proteins. There was no nuclear staining detected even when just the catalytic domain of PKG II was expressed in HEK cells (Fig. 5). This indicates that the catalytic domain does not translocate into the nucleus, unlike the PKG I catalytic domain, which has been shown to translocate into the nucleus in the absence of the N-terminus (Browning et al., 2001). This also indicates that the catalytic domain alone is unlikely to play a role in targeting of PKG II.

3. N-myr is important for targeting of PKG II to membranes and probable Golgi compartments in hippocampal neurons in culture.

Hippocampal neurons (10 *div*) were transfected with FLwt PKG II and FL PKG II G2A mutant using effectene reagent. Cells were fixed 15 *div* with 100% methanol and stained with rabbit GFP antibody and Alexa Fluor 488 anti-rabbit IgG secondary antibody to visualize the recombinant proteins. Fig. 16a shows that FLwt PKG II was localized predominantly in the perinuclear region, just like in COS-1 and HEK-293 cells. In contrast FL PKG II G2A mutant has a much more diffuse localization in the cell body of hippocampal neurons in culture (Fig. 16b). In the dendrite FLwt PKG II has a punctate distribution, which is consistent with membrane association (Fig. 16c). FL PKG II G2A mutant is diffusely localized even in the dendrites (Fig. 16d). Neither did we observe nuclear localization of the recombinant PKG II proteins in hippocampal neurons.

These results indicate that N-myr is important for targeting of PKG II to the membrane and other subcellular sites in COS-1 cells, HEK cells and hippocampal neurons in culture.

Figure 15: Immunocytochemical localization of various recombinant PKG II proteins and GFP in HEK-293 cells.

HEK-293 cells were transfected with various PKG II GFP constructs using Lipofectamine reagent. Cells were fixed 24 hours post transfection with 2% PFA and stained with rabbit GFP antibodies and corresponding fluorescent secondary antibodies. 5'wt PKG II transfected cells (a) show concentration of proteins in possible focal adhesion points and filopodia like structures. Fig. 15b shows a magnified portion of a. Arrowheads in b indicate concentration of protein in possible focal adhesion points and filopodia like structures. Fl wt PKG II also appears to concentrate in filopodia like structures (arrowheads in e). However the non-myristoylated PKG II mutants, 5' PKG II G2A mutant (Fig. 15 c&d), FL PKG II G2A mutant (Fig. 15 f) and GFP (Fig. 15 g) have a much more diffuse localization (Fig. 15 c, d, f and g). 20 cells from each transfection category from two different batches were analysed.
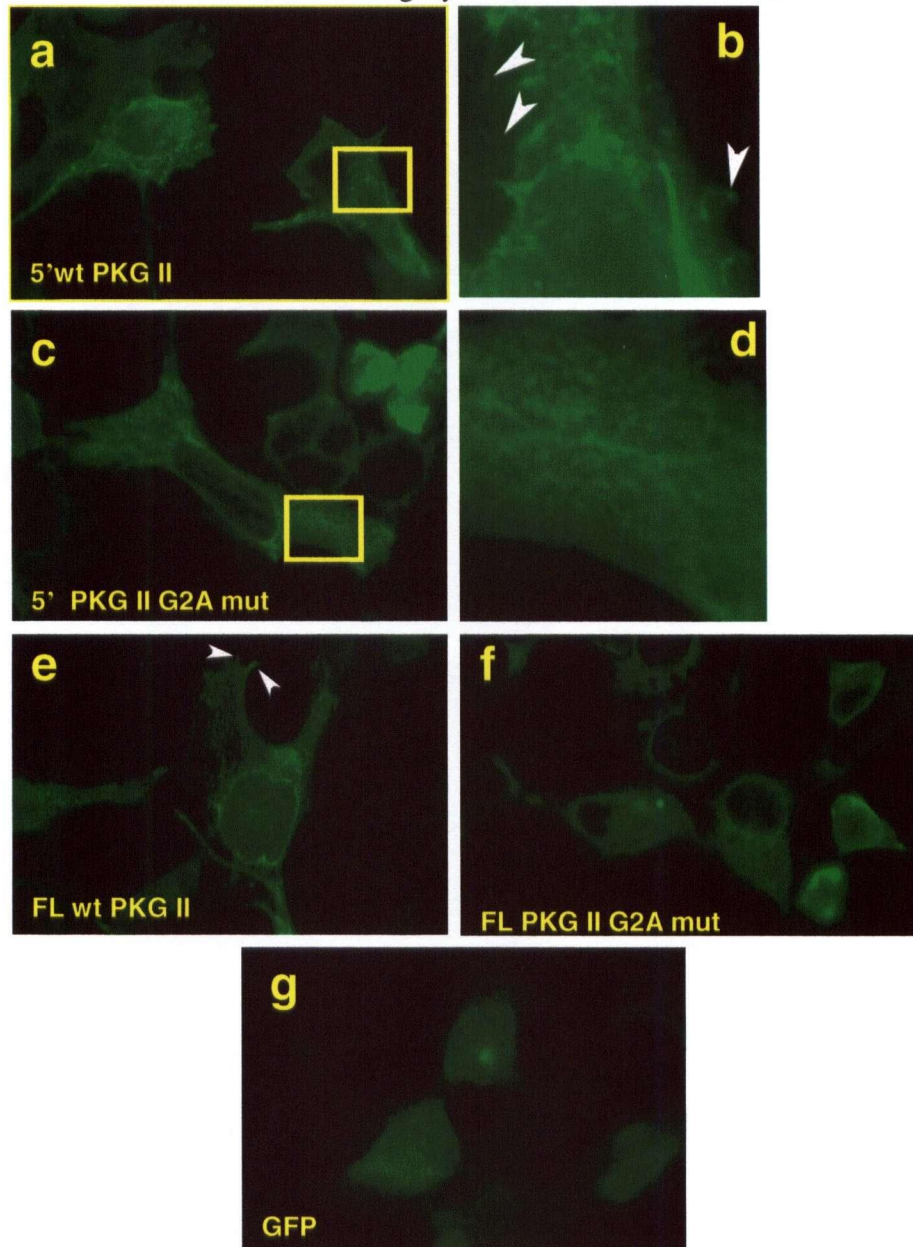
Figure 16: Immunocytochemical localization of FLwt PKG II GFP and FL PKG II G2A mutant GFP in hippocampal neurons in culture.

Expression of FLwt PKG II GFP (a) and FL PKG II G2A mutant (b) in the cell body of hippocampal neurons in culture and a magnified process in hippocampal neurons in culture (c and d) is shown. Arrows in c indicate membranous staining and arrowheads shows concentration of the protein in tips of spine like structures. At least 10 cells of each transfection category from two different batches of transfection were analysed.
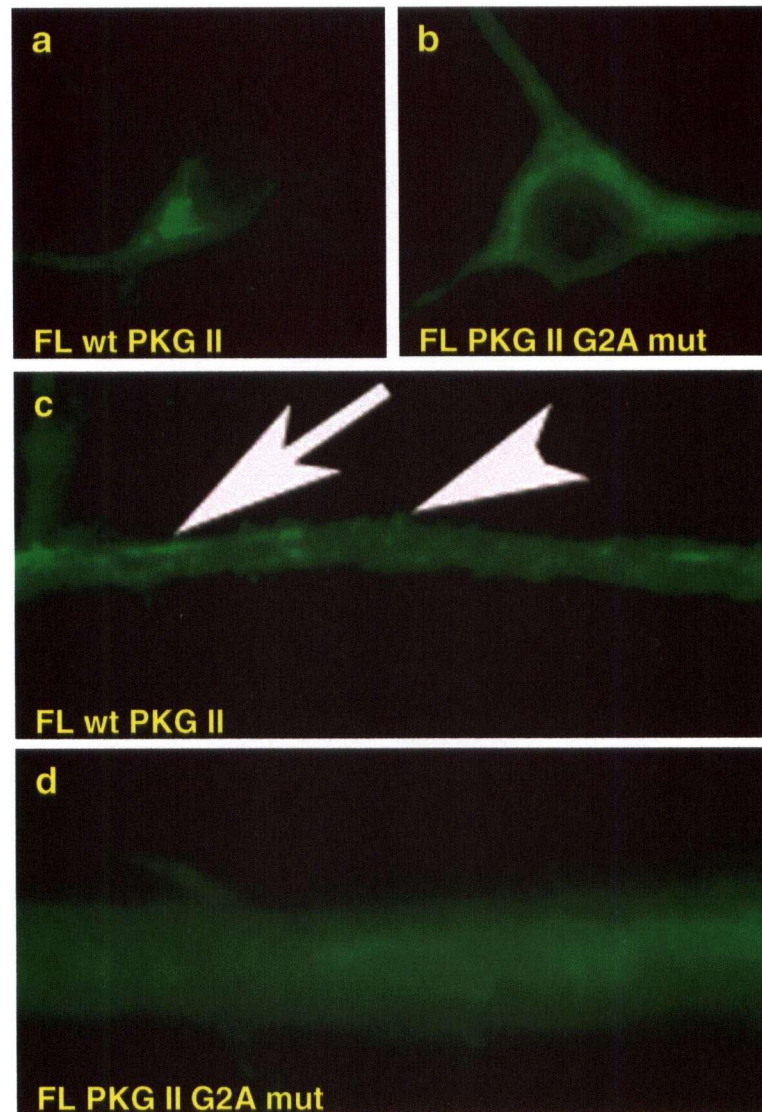
3. N-myr appears to be important for synaptic targeting of PKG II GFP in hippocampal neurons in culture.

Based on the punctate localization of FLwt PKG II in hippocampal neurons in culture, we wanted to test if any of these puncta corresponded to synaptic sites and if myristoylation was important for targeting of FLwt PKG II to synaptic sites in hippocampal neurons in culture. Ten *div* hippocampal neurons in culture were transfected with FLwt PKG II and FL PKG II G2A mutant using effectene reagent. Cells were fixed 15 *div* with 100% methanol and double stained with a mouse GFP antibody and rabbit synaptophysin antibody. FITC conjugated anti-mouse IgG and Texas-red conjugated anti-rabbit IgG secondary antibodies were used to visualize the recombinant proteins. Fig. 17 shows a dendritic process of a hippocampal neuron in culture overexpressing FLwt PKG II GFP (Fig. 17a left panel) and FL PKG II G2A mutant GFP (Fig. 17a top right panel, endogenous synaptophysin (Fig. 17b right and left panel) and the merged images (Fig. 17c right and left panel). A portion of this dendritic branch is magnified in Fig. 18. It is clear that FLwt PKG II had a punctate distribution (Fig. 17a left panel) and some of these puncta partially co-localized with the presynaptic molecular marker synaptophysin (Fig. 18c right panel), indicating that these were synaptic sites. FL PKG II G2A mutant by contrast had a diffuse distribution along the dendrite (Fig. 18a right panel) and there was no apparent concentration of the FL PKG II G2A mut protein at synaptic sites as shown by synaptophysin staining (Fig. 18c right panel). **This demonstrated that N-myr is important for targeting FLwt PKG II to synaptic sites in hippocampal neurons.**

Figure 17: Myristoylation is important for synaptic targeting of FL PKG II GFP in hippocampal neurons in culture.

Hippocampal neurons (10 *div*) were transfected with FLwt PKG II GFP and FL PKG II G2A mutant GFP using Effectene reagent. Cells were fixed 15 *div* with methanol and double stained with rabbit anti-GFP and mouse anti-synaptophysin and corresponding fluorescent secondary antibodies. Arrowheads indicate synaptic contacts. At least 10 cells of each transfection category from two different batches of transfection were analysed.
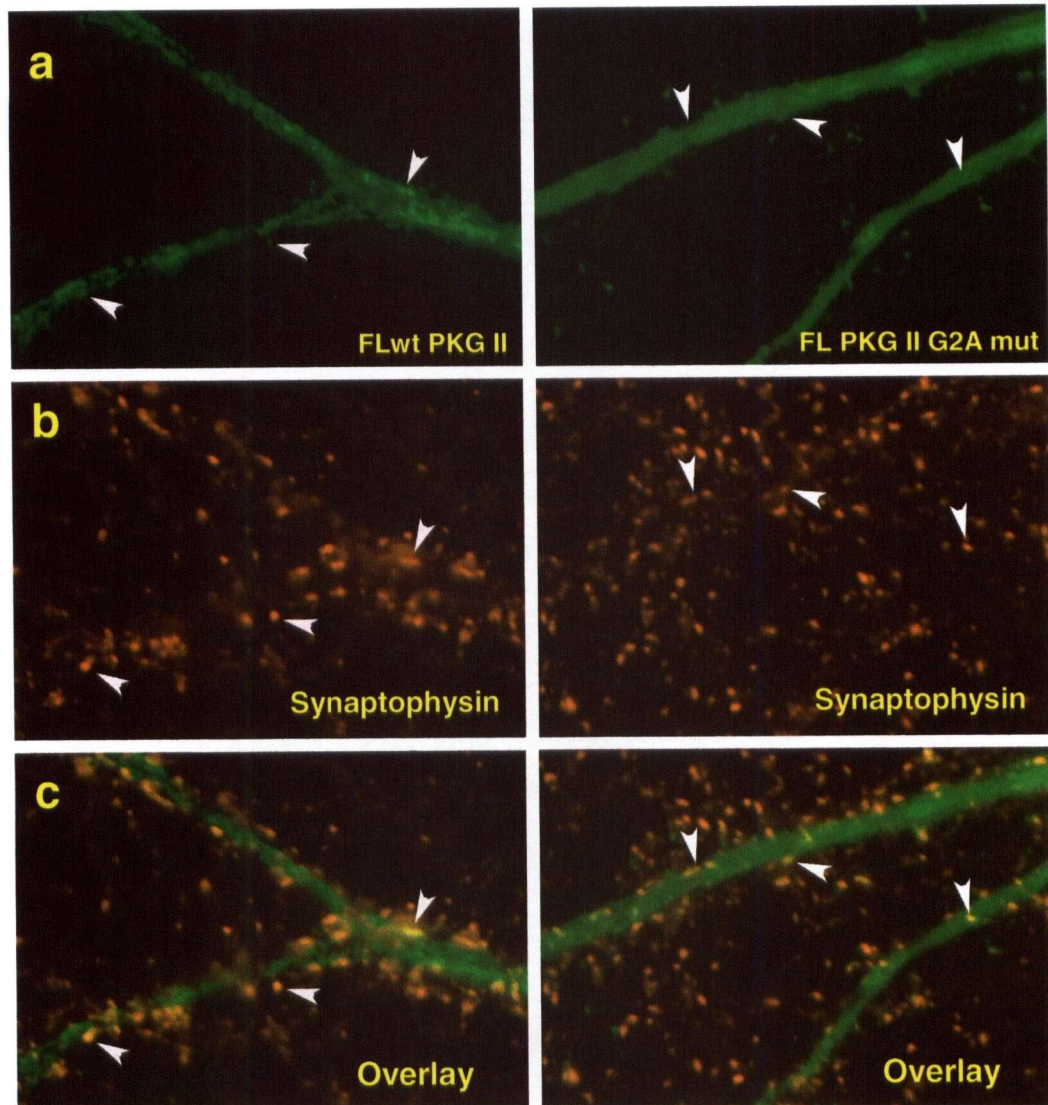
Figure 18: Myristoylation is important for synaptic targeting of FL PKG II GFP in hippocampal neurons in culture.

A high magnification of a dendritic process is shown. FLwt PKG II has a punctate distribution (a left panel) as compared to the diffuse distribution of FL PKG II G2A mutant. FLwt PKG II appears to be concentrated at synaptic sites (c left panel) as compared to FL PKG II G2A mutant protein (c right panel). FL PKG II is clearly clustered at sites where there is synaptophysin staining. Such an accumulation of FL PKG II G2A mutant is not observed.
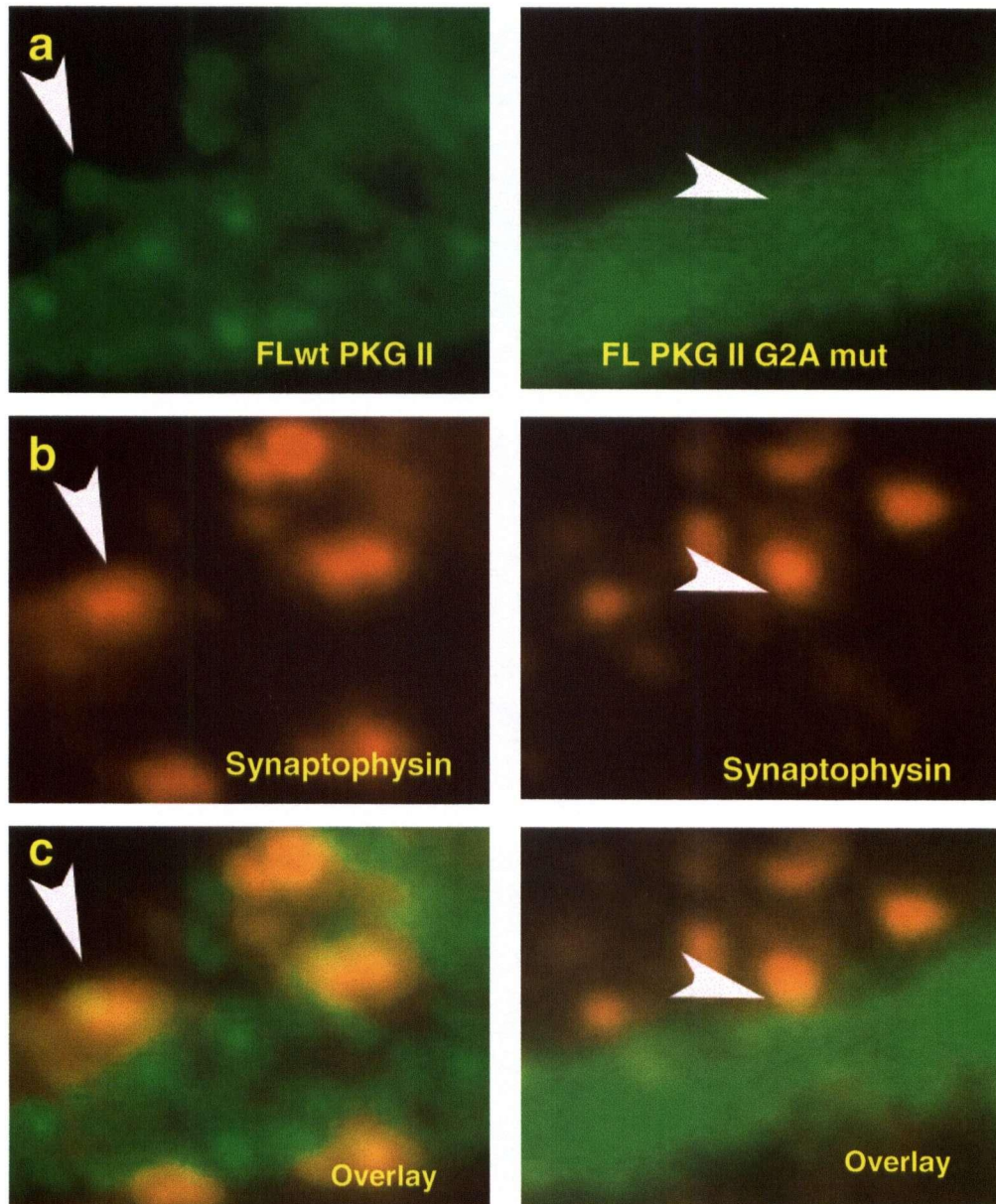
IV. Function of PKG II in hippocampal neurons.

1. 5'wt PKG II inhibits endogenous PKG I activitiy in HEK-293 cells.

Recently a novel splice variant of PKG II (PKG II $\Delta^{441-469}$) lacking 29 amino acids of the PKG II Mg-ATP binding/catalytic domain was discovered (Gambaryan et al., 2002). Gambaryan et al. showed that this isoform of PKG II does not have intrinsic enzymatic activity itself, but can antagonize PKG II and PKG I but not PKA activity. This is the first evidence for the presence of an endogenous inhibitor of PKG II. Thus PKG II function can not only be regulated by cGMP levels, but also by this novel splice isoform, which has been shown to act as a dominat negative.

We wanted to first test if our PKG II deletion mutant 5'wt PKG II can inhibit endogenous PKG I activity in HEK cells, like the splice isoform of PKG II as previously shown by Gambaryan et al. using PKG II $\Delta^{441-469}$ (Gambaryan et al., 2002). **VA**sodilator **S**timulated **P**hospho-Protein (VASP), which has been shown to be a PKG substrate, was used in the phosphorylation studies. We co-transfected HEK cells with either GFP and human VASP or 5'wt PKG II GFP and human VASP. Twenty four hours post-transfection cells were treated with 8Br-cGMP for 15 min. Cells were lysed and SDS-PAGE was performed followed by Western blotting using a phospho-Ser^{239} VASP specific antibody, which detects VASP phosphorylated on Ser^{239}, which is the preferred site of phosphorylation for PKG. As shown in figure 19b (n=4) there is a clear reduction in the Ser^{239} phosphorylated VASP in cells overexpressing 5'wt PKG II as compared to cells expressing GFP. Fig. 19a shows that GFP and 5'wt PKG II GFP proteins were expressed. A monoclonal antibody that detects

phosphorylated and non-phosphorylated VASP was used to make sure that equal amounts of VASP proteins were loaded (Fig. 19c). Finally in order to detect endogenous PKG I, a PKG I specific antibody was used to confirm PKG I was expressed in HEK cells. Gambaryan et al. previously showed that PKG II $\Delta^{441-469}$ markedly inhibited the activity of wt PKG II and PKG I in NIH-3T3 cells stably transfected with wt PKG II and wt PKG I, respectively (Gambaryan et al., 2002). We have shown that the regulatory domain of PKG II when expressed alone, like PKG II $\Delta^{441-469}$, can inhibit endogenous PKG I activity in HEK-cells. Based on this data and the study by Gambaryan et al., it is plausible to assume that overexpressing 5'wt PKG II GFP in cells expressing PKG II would inhibit the endogenous PKG II activity as well. We thus wanted to use the 5'wt PKG II construct as a possible dominant negative in hippocampal neurons to study the function of PKG II.

Figure 19: wt regulatory domain of PKG II reduces phosphorylation of VASP in HEK-293 cells.

HEK-293 cells were transfected with either 5'wt PKG II GFP and human VASP or GFP and human VASP using lipofectamine reagent. VASP was used for assessing its phosphorylation as a measure of PKG II activity. Twenty four hours post transfection cells were treated with 50 μM 8-Br cGMP. Cells were lysed and Western blotting was performed using GFP antibodies (a), phospho-Ser^{239} VASP antibody that detects only the Serine 239 phosphorylated form of VASP (b), mouse monoclonal VASP antibody (c) and PKG I antibody (d). There is a clear reduction in Ser^{239} phosphorylated form of VASP upon 8-Br cGMP treatment in wt regulatory domain expressing cells (b right panel) as compared to GFP expressing cells (b left panel), (n=3).
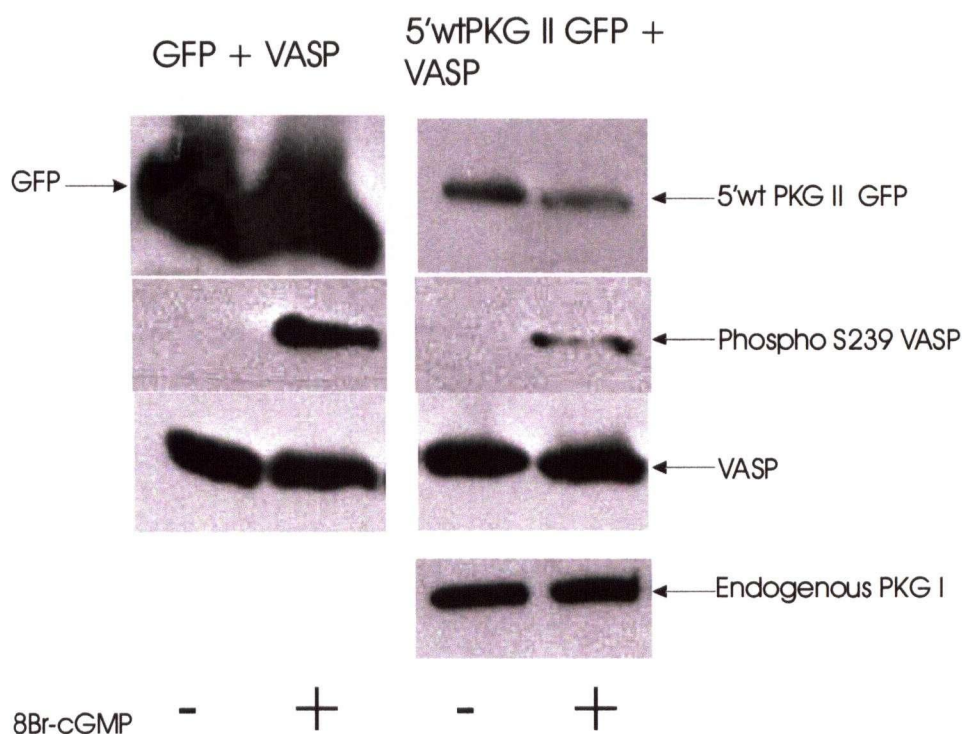
2. Hippocampal neurons as a model for studying PKG II function.

Hippocampus has been one of the best models for studying plasticity in the brain. Both forms of synaptic plasticity, LTP and LTD, have been extensively studied in both hippocampal slices and hippocampal neurons in culture. There is considerable evidence supporting the role of the NO/cGMP/PKG pathway in LTP and LTD in the hippocampus. We have also shown in this study that PKG II is present endogenously in hippocampal neurons in culture and also localizes to synaptic sites. Hence we chose to use hippocampal neurons in culture as a model for studying the function of PKG II.

Most of the studies implicating a role for PKG II in neuronal function (see Introduction) were done using a selective PKG inhibitor such as KT-5823. However, it was recently shown that KT-5823 did not inhibit PKG in a standard protein kinase assay (Bain et al., 2003). Bain et al also showed that KT-5823 inhibited PRAK and GSK 3β *in vitro* by 50% at a concentration of 10 μM. There was also another report that indicated that KT-5823 does not inhibit PKG in intact human platelets or mesangial cells (Burkhardt et al., 2000). Based on these studies it appears that KT-5823 is not a very selective PKG inhibitor. To avoid any discrepancy due to possible cross reactivity of PKG inhibitors, we chose to study the function of PKG II by overexpressing a dominant negative form of PKG II in hippocampal neurons instead of using the commercially available PKG inhibitors. We wanted to find "loss of function" phenotypes in cultured hippocampal neurons transfected with just the regulatory domain of PKG II (5'wt PKG II), which is a possible dominant negative form of PKG II.

3. Overexpression of regulatory domain of PKG II in 10 *div* hippocampal neurons caused a 2.5-fold increase in filopodia-like structures compared to GFP overexpressing neurons.

Neurons transfected with 5'wt PKG II GFP (Fig. 20b, 21) showed a striking increase in density of filopodia compared to control GFP transfected neurons (Fig. 20a, Fig. 21). In contrast the FLwt PKG II (Fig. 20c) and the non-myristoylated forms of PKG II, 5'PKG II G2A mutant (Fig. 20d) and FL PKG II G2A mutant (Fig. 20e) had similar number of filopodia as GFP overexpressing cells (Fig. 21). To visualize the entire cell we co-transfected the PKG II constructs with an RFP construct (Fig. 24). Fig. 24 shows that the fewer filopodia seen in FLwt PKG II and the non-myristoylated PKG II overexpressing cells is not due to improper targeting of the recombinant proteins and lack of visualization of the dendritic protrusions. This also indicated that N-myr is important for the regulatory domain of PKG II to cause an increase in number of filopodia as the non-myristoylated regulatory domain did not show an increase in density of filopodia.

Figure 20: Exogenous PKG II regulatory domain led to a 2.5-fold increase in number of filopodia in 10 *div* hippocampal neurons in culture.

Six *div* hippocampal neurons were transfected with various PKG II constructs and stained for GFP at 10 *div*. Examples of dendrites transfected with GFP (a), wt regulatory domain of PKG II (b), FL wt (c), non-myristoylatted regulatory domain (d) and non-myristoylated FL PKG II (e) are shown. As shown, there was a dramatic increase in filopodial processes in the wt regulatory domain transfected cells. Atleast 15 cells in each transfection category from three different batches were analysed. Dendritic protrusions on every dendrite of each cell were counted.
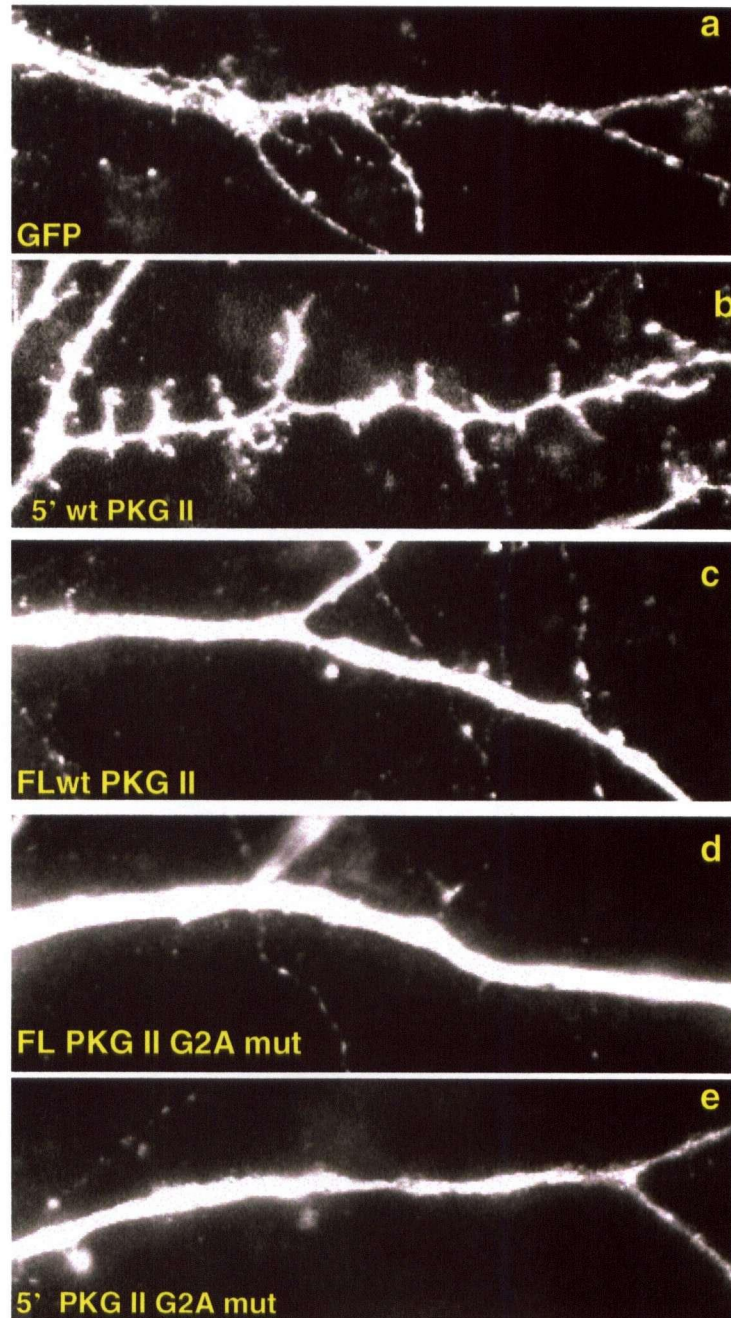
Figure 21: Exogenous PKG II regulatory domain led to a 2.5-fold increase in number of filopodia in 10 *div* hippocampal neurons in culture.

Data analysis was performed using Northern Eclipse program (see details in Materials and Methods). Number of protrusions from the dendrites were counted and automatically logged into Microsoft Excel. Error bars represent standard deviation. 5'wt PKG II overexpressing cells show a significant increase in density of filopodia ($p < 0.0008$).
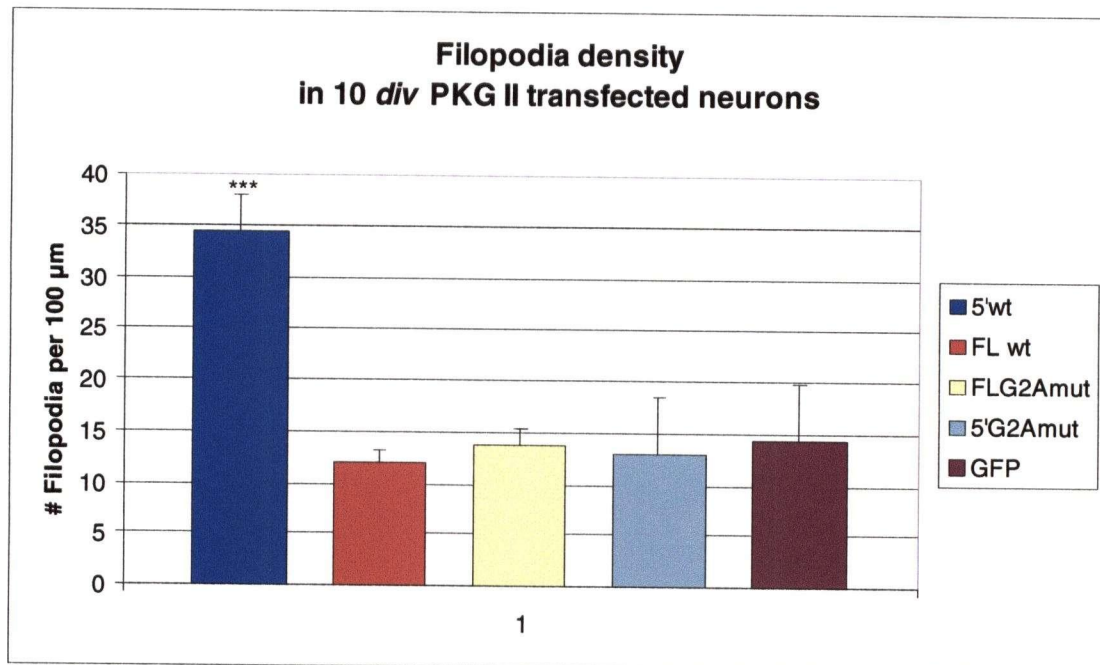
4. Overexpression of regulatory domain of PKG II in 15 *div* hippocampal neurons caused a 2-fold increase in spine like structures compared to GFP overexpressing neurons.

Based on the data obtained from the previous study, we wanted to assess if the increase in filopodia translated to increase in spines in older cultures. Neurons transfected with 5'wt PKG II GFP (Fig. 22b, 23) showed a striking increase in density of spine-like structures compared to control GFP transfected neurons (Fig.22a, Fig. 23). In contrast, the FLwt PKG II (Fig. 22c) and the non-myristoylated forms of PKG II, 5'PKG II G2A mutant (Fig. 22d) and FL PKG II G2A mutant (Fig. 22e), had a similar number of spine-like structures as GFP overexpressing cells (Fig. 23). As shown in Fig. 17 and 18, N-myr is important for proper targeting of PKG II to membranes and synaptic sites. As in the case of 10 *div* hippocampal neurons, N-myr appears to be important for the regulatory domain of PKG II to cause an increase in number of spine-like structures as the non-myristoylated regulatory domain did not show an increase in density of spine like structures.

Figure 22: Exogenous PKG II regulatory domain led to a 2-fold increase in number of spine-like structures in 15 *div* hippocampal neurons in culture.

Ten *div* hippocampal neurons were transfected with various PKG II constructs and stained for GFP at 15 *div*. Examples of dendrites transfected with GFP (a), wt regulatory domain of PKG II (b), FL wt (c), non-myristoylatted regulatory domain (d) and non-myristoylated FL PKG II (e) are shown. As shown, there was a drastic increase in spine like structures in the wt regulatory domain transfected cells. Atleast 15 cells in each transfection category from three different batches were analysed. Dendritic protrusions on every dendrite of each cell were counted.
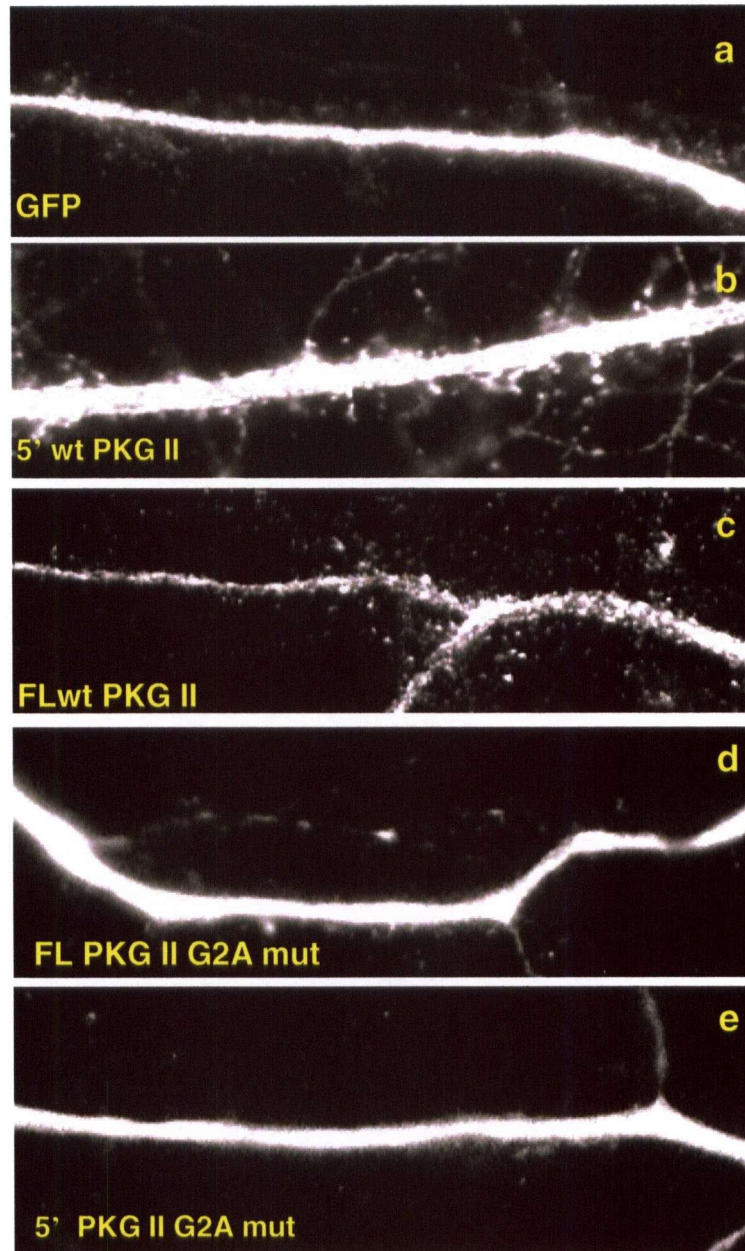
Figure 23: Exogenous PKG II regulatory domain led to a 2-fold increase in number of spine-like structures in 15 *div* hippocampal neurons in culture.

Data analysis was performed using Northern Eclipse program (see details in materials and methods). Number of protrusions from the dendrites were counted and automatically logged into Microsoft Excel. Error bars represent standard deviation. As in the case of 10 *div* 5'wt PKG II overexpressing cells show a significant increase in density of spine-like structures (p<0.0001).
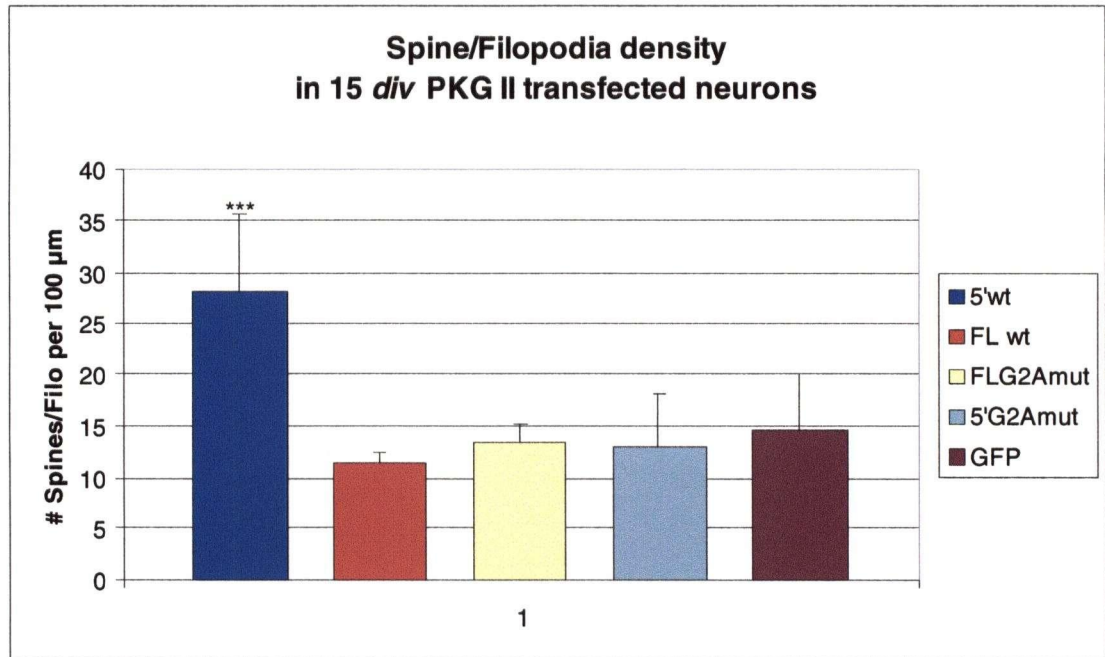
Figure 24: Hippocampal neurons transfected with non-myristoylated forms of PKG II do not show an increase in density of filopodia/spines.

Six *div* hippocampal neurons were co-transfected with various PKG II constructs and RFP and fixed with 2% PFA at 10 *div*. Examples of dendrites transfected with wt regulatory domain of PKG II, FL wt, non-myristoylated regulatory domain and non-myristoylated FL PKG II (Panel A), RFP (Panel B) and merged images (Panel C) are shown. As shown, there is drastic increase in filopodial processes in the wt regulatory domain transfected cells and not in non-myristoylated forms or FL wt PKG II transfected neurons. Atleast 10 cells in each category from two different batches were analysed.
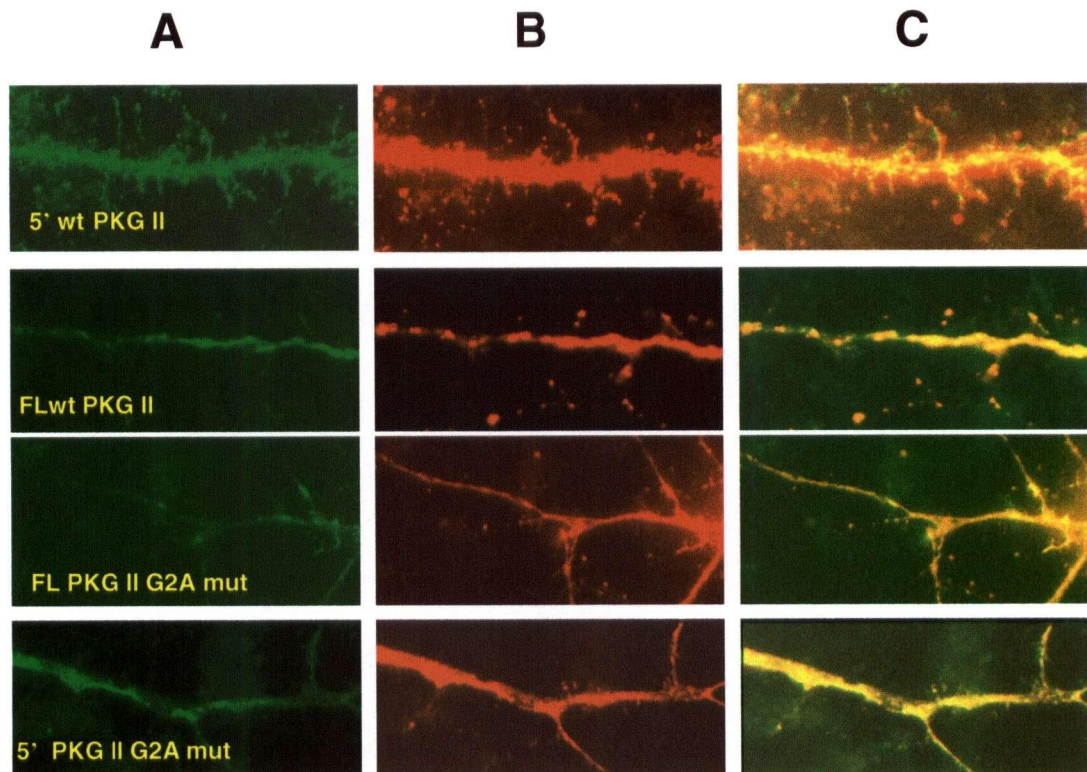
5. Most of the dendritic protrusions in the wt regulatory domain PKG II transfected neurons appear to be synaptic.

To find out if the dendritic protrusions might correspond to synaptic sites, we co-stained hippocampal neurons transfected with 5'wt PKG II with GFP and synaptophysin antibodies (Fig. 25A) or GFP and PSD-95 antibodies (Fig. 25B). Fig 25C shows that more than 90% of the dendritic protrusions in the 5'wt PKG II overexpressing cells co-localized with both the presynaptic marker synaptophysin and the postsynaptic marker PSD-95. This shows that there was an increase in synaptic contacts in the PKG II dominant negative overexpressing neurons and most of the increase in filopodia seen during 10 *div* appeared to go on to become spines.

Figure 25: Dendritic protrusions in wt regulatory domain transfected cells co-localized with the presynaptic marker synaptophysin and the postsynaptic marker PSD-95.

Ten *div* hippocampal neurons were transfected with wt regulatory domain of PKG II construct and stained for GFP, synaptophysin (A) and PSD-95 (B) at 15 *div*. Most of the protrusions from the dendrite of a 5'wt PKG II overexpressing cell co-localize with the synaptic markers, indicating that they are most likely to be functional synapses. Atleast 10 cells in each category from two different batches were analysed.
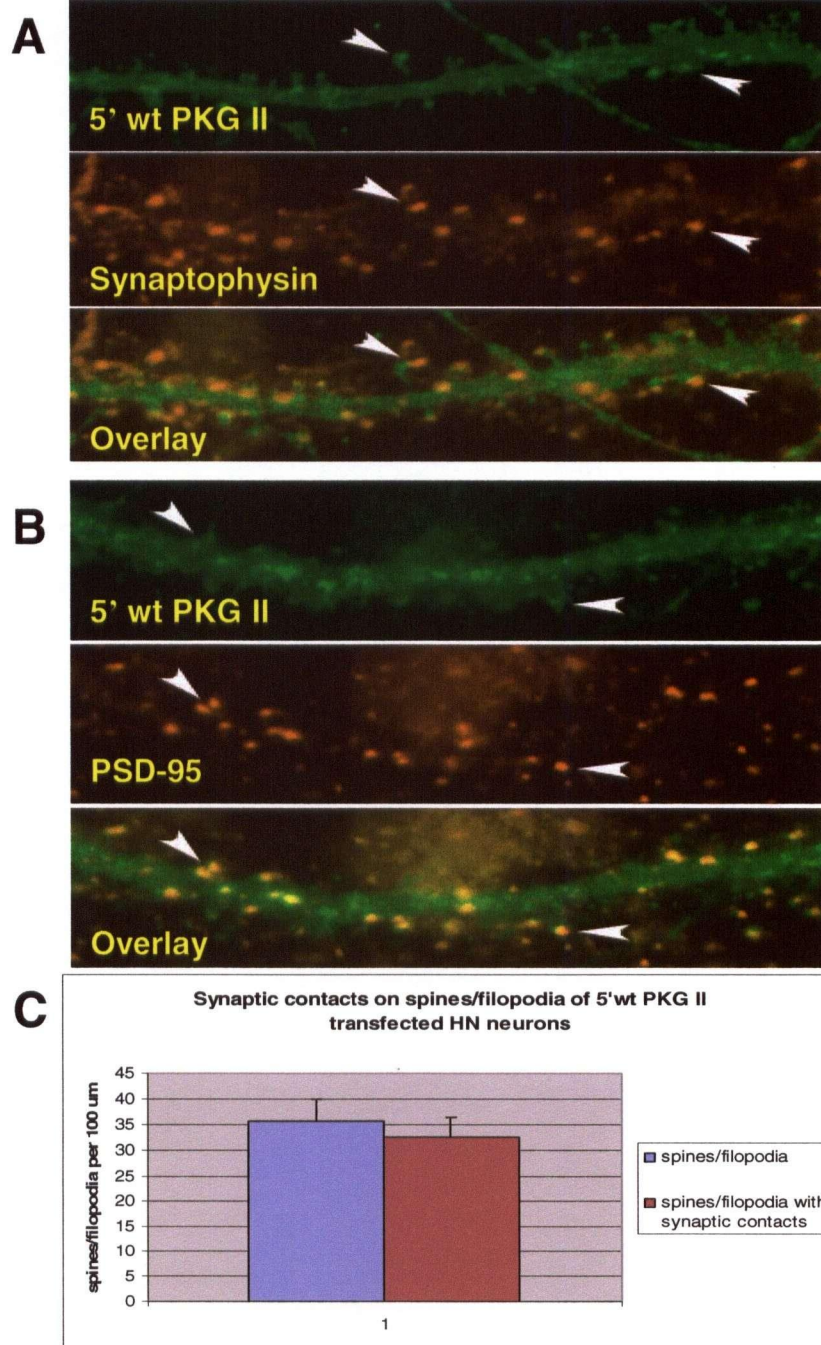
6. There was a corresponding increase in density of synaptophysin puncta in 5'wt PKG II overexpressing hippocampal neurons.

As most of the spine like structures in the 5'wt PKG II overexpressing neurons co-localized with the synaptic markers, we also wanted to analyze if there was a corresponding increase in the presynaptic marker synaptophysin puncta. Fig. 26 shows that this is the case as there is a 2-fold increase in synaptophysin puncta in 5'wt PKG II overexpressing neurons. This indicates that the increase in filopodia translates to probable functional synapses.

7. Overexpression of human form of VASP had no effect on morphology of hippocampal neurons and does not show an increase in density of synaptic contacts.

Cell crawling is an important phenomenon that drives processes such as morphogenesis and metastasis. Cell locomotion is associated with actin polymerization (Mogilner and Oster, 1996; Mogilner and Oster, 2003). The increase in density of spines and filopodia like structures has also been shown to involve actin polymerization (Fischer et al., 1998). A recent model for cell motility suggests a possible motor-like mechanism based on the modulated binding interaction between actin filaments and Vasodialor stimulated phosphoprotein (VASP), which is fueled by hydrolysis of actin-bound ATP (Dickinson and Purich, 2002).. VASP is a well known PKG substrate (Waldmann et al., 1986), that induces polymerization of G-actin into F-actin bundles in *in vitro* assays and it is thought to stabilize F-actin in a phosphorylation dependent manner (Laurent et al., 1999). Excess of VASP results in long, unbranched filaments in fibroblasts (Bear et al., 2002). VASP has been shown to be expressed in neonatal brain but not in adult brain and has been implicated to play a role in development (Gambaryan et al., 2001). We wanted to test if overexpression of

VASP could lead to an increase in filopodia in neurons. Human VASP construct (gift from Dr. Ulrich Walter) was transfected into 10 *div* hippocampal neurons using Effectene reagent. Cells were fixed 4-5 days later with 100 % methanol and visualized using a monoclonal antibody against VASP. Cells were also stained with the presynaptic marker protein synaptophysin to find out if there is an increase in number of synaptic contacts on VASP overexpressing cells. Phosphorylation of VASP has been shown to negatively regulate actin polymerization (Harbeck et al., 2000; Walders-Harbeck et al., 2002). As VASP is a substrate of PKG, we treated cells for 4-5 days with 100 µM of the PKG selective activator 8Br-cGMP to see if phosphorylation of VASP by endogenous PKG II has any effect on the morphology of neurons. As shown in Fig. 27, there was no apparent increase in density of synaptic contacts, as shown by synaptophysin puncta, in untreated VASP overexpressing cells (Fig. 27A, 27C) when compared to GFP overexpressing cells (Fig. 27C) or VASP overexpressing cells treated with 8Br-cGMP (Fig. 27 B, 27C).

Figure 26: There is a 2-fold increase in synaptophysin puncta in 5'wt PKG II overexpressing neurons.

Ten *div* hippocampal neurons were transfected with various PKG II constructs and stained for GFP and synaptophysin at 15 *div*. Length of dendrites was manually traced and number of synaptophysin puncta on dendrites were manually counted and entered into Microsoft Excel. Error bars represent standard deviation. As in the case of 15 *div* 5'wt PKG II overexpressing cells show a significant increase in desity of synaptophysin puncta (p<0.0001). Atleast 10 cells in each category from two different batches were analysed.
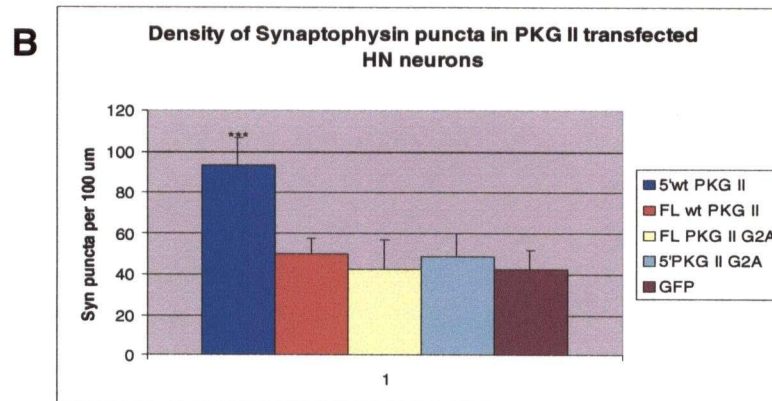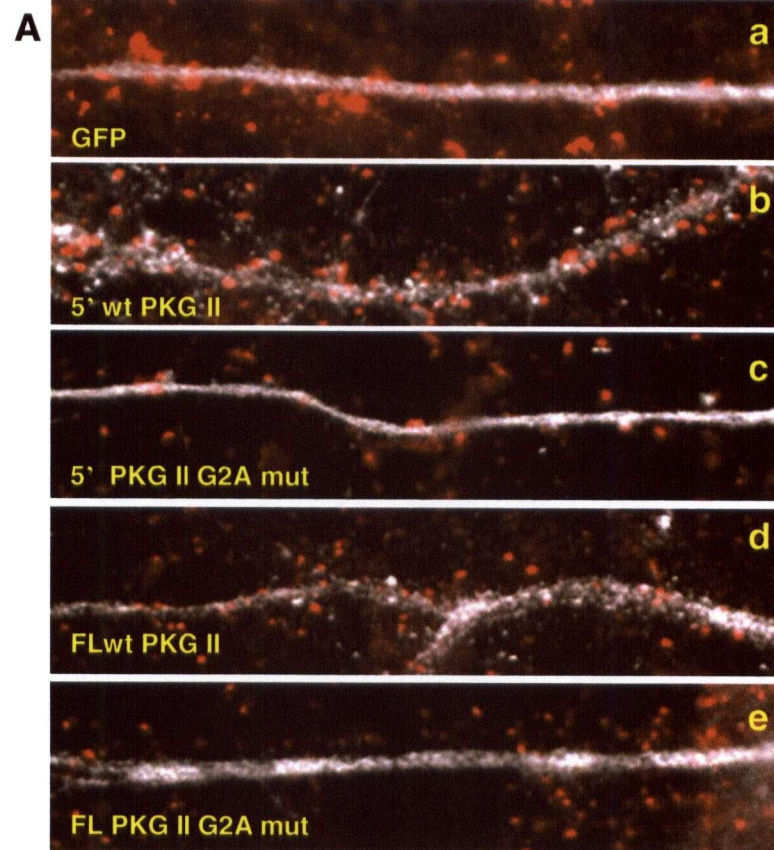
Figure 27: Overexpression of human VASP in neurons did not show an increase in density of synaptophysin puncta.

Human VASP or GFP constructs were transfected into hippocampal neurons on 10 *div* using Effectene reagent and fixed 15 *div* using 100% methanol. VASP overexpressing neurons were either untreated or treated with 8Br-cGMP for four days. There is no apparent increase in density of synaptophysin puncta in untreated VASP overexpressing neurons (Fig. 26 A & C) when compared to either GFP (Fig. 26 C) or VASP overexpressing neurons treated with 8Br-cGMP (Fig. 26 B & C). Error bars represent standard deviation. Results are a representation of four different expreriments. Atleast 10 cells in each category from two different batches were analysed.

VASP wt + Synaptophysin

VASP wt + Synaptophysin + 8Br-cGMP

C

Density of Synaptophysin puncta in VASP transfected HN neurons

Legend:
- VASPwt No treat
- VASPwt 8Br-cGMP
- GFP
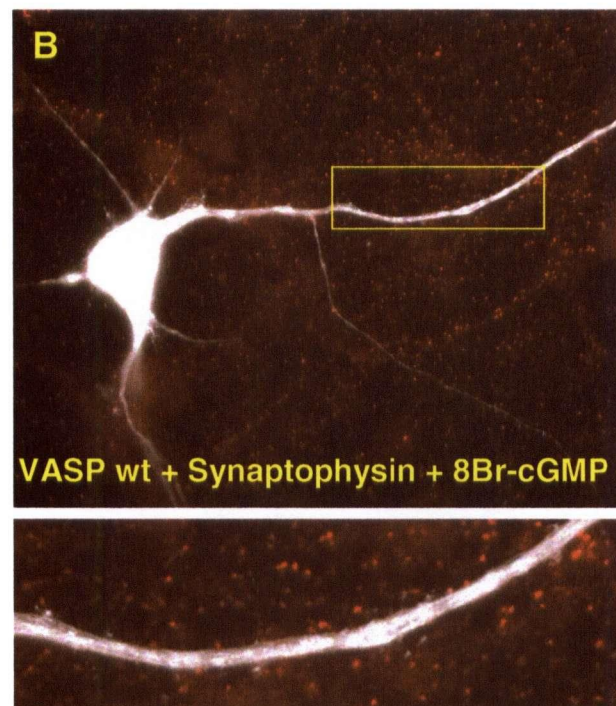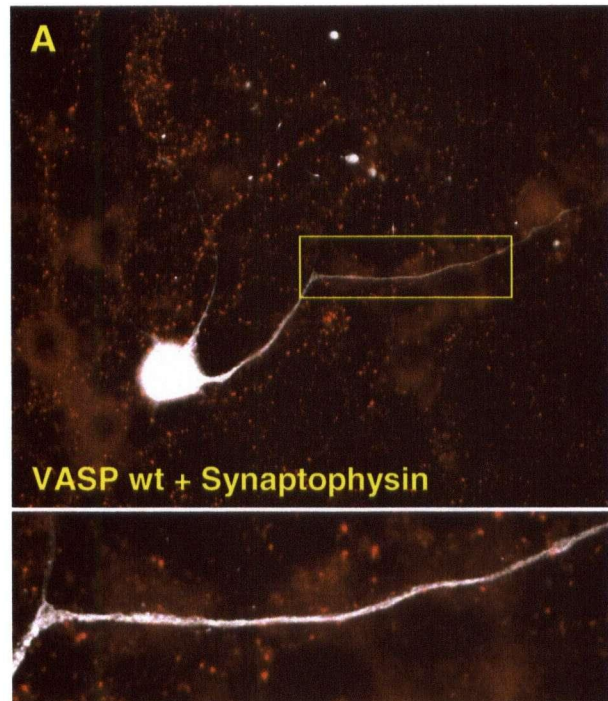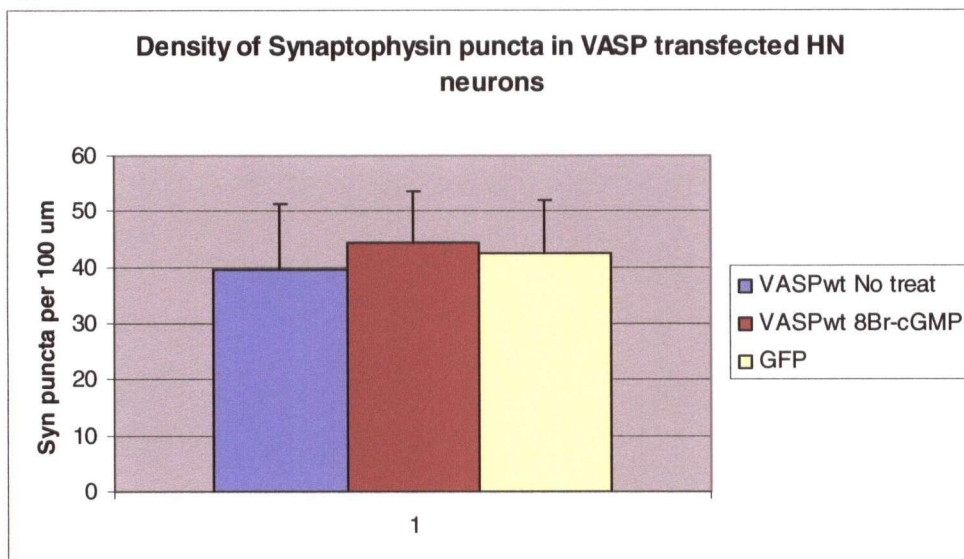
Discussion

PKG is the major downstream effector of the NO/cGMP and natriuretic peptides/cGMP pathway. Recent evidence indicates that PKG mediates a number of neuronal effects of cGMP, but how it brings about its effect is still unclear. Of the three PKG isoforms known so far, PKG II is much more widely distributed than PKG Iα and PKG Iβ. Although the distribution of PKG II in various brain regions is known, the subcellular localization of PKG II and the regions of PKG II protein responsible for its subcellular localization in the brain are poorly understood. In spite of the widespread expression of PKG II, its function in the brain is still unknown.

This thesis involved characterization of a PKG II specific antibody, examination of the subcellular distribution of PKG II in the brain, evaluation of the importance of N-myr in the subcellular distribution of PKG II, and investigation of possible role for PKG II in synaptogenesis.

I. Characterization of PKG II antibody.

A polyclonal antibody against PKG II was raised using a 13 amino acid peptide corresponding to the hinge region of the rat PKG II sequence. The hinge region, between the second cGMP binding site and the start of the Mg-ATP binding site, exhibits the highest sequence divergence from the other PKG isoforms. Hence, this region was used to raise an antibody as it is most likely to be specific to PKG II.

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1. Antibody specificity

The specificity of the antibody was tested in a number of different ways. The PKG II antibody recognized only the FLwt PKG II recombinant protein expressed in HEK-293 cells and not the regulatory domain of PKG II (5'wt PKG II), which lacks the antibody recognition site. HEK-293 cells expressed PKG I (Fig. 19d). However, our PKG II antibody did not recognize any band in untransfected or 5'wt PKG II expressing HEK-293 cell lysates, which indicated that the PKG II antibody did not crossreact with PKG I. The affinity purified antibodies immunoprecipitated a major band of ~86 kD in brain lysates and lysates from hippocampal neurons in culture. When these antibodies were omitted or a PKG I specific antibody was used, no bands were detected in the immunoprecipitation studies. PKG II antibody stains hippocampal, cortical and thalamic neurons in culture and this staining was abolished when the antibody was preadsorbed with the peptide against which it was raised prior to performing immunocytochemistry.

2. Distribution of PKG II in the brain and comparison with other PKG II localization studies.

Western blot analysis using the PKG II antibody revealed a ~86 kD band in the lysates of cortex, hippocampus, hypothalamus, striatum and thalamus. No such band was detected in cerebellar lysates. Our results of high PKG II expression in the rat brain was in agreement with the PKG II mRNA distribution study by El-Husseini et al. (el-Husseini et al., 1995; El-Husseini et al., 1999) and other reports of high levels of PKG II in the rat and mouse brain (Jarchau et al., 1994; Lohmann et al., 1997; Uhler, 1993). El-Husseini et al. reported very

low levels of PKG II mRNA in cerebellum which correlated with our Western blotting analysis of cerbellar lysates using PKG II antibody, where we did not detect any bands. Others have shown low levels of PKG II in the cerebellum by Western blotting analysis. However, they detected much stronger immunocytochemical PKG II staining in the cerebellum (de Vente et al., 2001). El-Husseini et al., showed low levels of PKG II mRNA in striatum and hippocampus and highest levels in the thalamus. But our study using PKG II antibody detected highest levels of PKG II protein in the cortex and striatum and high levels of PKG II protein in the hippocampus and thalamus, which does not coincide with the study by El-Husseini et al. An earlier report, however, indicated the presence of PKG in the medium spiny neurons (Ariano, 1983) and de Vente et al. reported the presence of PKG II in the striatum (de Vente et al., 2001). de Vente et al. did not find that the cell bodies were stained in the striatum. Striatum receives input from midline thalamic nuclei and the subthalamic nucleus, which express high levels of PKG II. Our results of high PKG II expression in the striatum could be partially explained by the probable presence of PKG II in the nerve terminals of these inputs. de Vente et al. reported low levels of PKG II in the hippocampus. Hippocampus receives input from the cortex, which has high levels of PKG II and again it is tempting to speculate that the high protein expression observed in the hippocampus in this study could be partly due to the protein present in the nerve terminals of cortical inputs. Our PKG II antibody stains hippocampal neurons in culture and PKG II appears to be present in both the cell body and dendritic processes and de Vente et al. also reported PKG II staining in some pyramidal neurons. On the one hand de Vente et al. rarely detected cell body staining in thalamic and subthalamic nuclei. Our results on the other hand show the presence of PKG II in the cell body and dendrites of thalamic neurons in culture. It

is unclear as to why this difference in staining was observed. Overall, the widespread distribution of PKG II in the brain is undisputed. Compared to PKG II, PKG I is highly expressed in the cerebellum, moderately expressed in the hypothalamus and very weakly expressed in the hippocampus and cortex. However, Arancio et al. showed that PKG I is highly expressed in the hippocampus and showed the presence of PKG I and not PKG II in hippocampal neurons in culture (Arancio et al., 2001). Using our PKG II antibody we found a high expression of PKG II in hippocampal neurons in culture, both in the cell body and dendritic processes. This discrepancy could be partially explained due to differences in affinities of antibodies used.

Based on these studies and studies mentioned above, it is clear that PKG II is highly expressed in the brain and has a much wider distribution in the brain than PKG I. This indicates that PKG II is most likely to be the downstream mediator of cGMP actions in the brain. A role for cGMP and PKG has been shown at nerve terminals {Akamatsu, 1993 #302;Sistiaga, 1997 #304;Gray, 1999 #303}, but the localization of PKG I and II in the synapses has still not been demonstrated. Next we used our PKG II specific antibody to study the subcelluar distribution of PKG II in the rat brain subcellular fractions and hippocamapal neurons in culture.

II. Subcellular distribution of PKG II

de Vente et al. showed that PKG II was localized more in the processes than in the cell body (de Vente et al., 2001). However, they could not conclusively show the localization of PKG

II at nerve terminals. We chose to study the subcellular distribution of PKG II using the PKG II antibody on subcellular fractions of rat brain and hippocampal neurons in culture. We found that PKG II was highly concentrated in the synaptic vesicle fraction and we found it in high levels in the synaptosomal membrane fraction which contains the postsynaptic density (PSD) in adult brain subcellular fractions. It was also highly present in the other membrane rich fraction like the small compartments fraction, which contains light membranes. It had a partially similar subcellular fractionation profile to NR1 in this respect, which is also highly expressed in the small compartments fraction and synaptosomal membrane fraction. In contrast, PKG I appeared to be very weakly expressed in both the synaptic vesicle fraction and synaptosomal membrane fraction, indicating that most of the cGMP functions at the nerve terminal may be mediated by PKG II and not PKG I. We also found that PKG II was expressed in the synaptosomal membrane fraction in embryonic brain subcellular fractions. It shared this subcellular fractionation profile with PSD-95, a PSD enriched protein, which has been shown to be important for synapse maturation (El-Husseini et al., 2000). By contrast, PKG I was very weakly expressed in the synaptosomal membrane fraction in embryonic rat brain subcellular fractions and concentrated in the soluble fraction.

PKG II is present in both the cell body and dendritic processes in hippocampal neurons in culture. We showed that it partially co-localized with the presynaptic molecular marker synaptophysin and the postsynaptic molecular marker PSD-95. At higher magnification a slight shift in fluorescence was observed when hippocampal neurons were double stained with PKG II and synaptophysin, indicating that the proteins were present in the opposite compartments of the synapse. Such a shift was not noted in dendritic processes double

stained with PKG II and PSD-95 indicating that PKG II was present in spine like structures on the postsynaptic side.

The NO, cGMP and PKG pathway has been shown to be important for regulation of hippocampal neuronal LTP (Arancio et al., 1995; Boulton et al., 1995; Lu et al., 1999; Son et al., 1998; Wu et al., 1998; Zhuo et al., 1994a). However, in all the studies mentioned above (see introduction for detailed description), the importance of PKG was shown using activators or inhibitors of PKG. The presence of PKG in the synapse has not been demonstrated using immunocytochemical methods. Our Western blotting study indicated that PKG II was present at a high concentration in hippocampus. We also showed that PKG II was present at high levels in hippocampal neurons in culture and that it is targeted to synaptic sites. It has been recently shown that LTP and LTD in corticostriatal pathways are regulated by PKA and PKG in striatal neurons (Calabresi et al., 2000; Calabresi et al., 1999). Their finding is consistent with our observation that PKG II is present in both the cortex and striatum. Using Western blotting we detected very low levels of PKG I in cortex and hippocampus and did not detect PKG I in the striatum indicating that PKG II is most likely the molecule responsible for the cGMP actions in these areas.

PKG has been shown to be involved in regulation of neurotransmitter release. (Akamatsu et al., 1993; Gray et al., 1999; Sistiaga et al., 1997) Recently G-Septin and Septin 3, proteins highly concentrated in the nerve terminals, were shown to be phosphorylated by PKG (Xue et al., 2004; Xue et al., 2000). Mena, which is a substrate of PKG, is a cytoskeletal protein associated with neuronal growth cone activity responsible for axonal path finding. Mena

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deficient mice have defective axonal pathfinding (Lanier et al., 1999). Neural growth cone responses are converted from repulsion to attraction by cAMP and cGMP signaling (Song et al., 1998). Although a role for cGMP and PKG has been suggested at the presynaptic terminal, the presence of PKG in the presynaptic terminals has not been demonstrated. Using subcellular fractions from the brain and by Western blotting we showed that PKG II is highly concentrated in the synaptic vesicle fraction. PKG II was present at a much higher concentration in the synaptic vesicle fraction than PKG I, indicating that these effects are most likely mediated by PKG II and not PKG I. Our data that PKG II is localized in the presynaptic vesicle fraction suggests that PKG II could contribute to potentiation, possibly by altering presynaptic release of transmitters.

NO has been shown to act post-synaptically, either by enhancing LTP in some cases or by suppressing it in others (Ko and Kelly, 1999; Murphy and Bliss, 1999). Ko and Kelly (1999) showed that postsynaptic co-injection of the NOS inhibitor L-NAME with Ca(2+)/CaM blocked Ca(2+)/CaM-induced synaptic potentiation. Murphy and Bliss (1999) found that photolytically released NO (1-4.5 microM) from bath applied caged NO reduced the magnitude of long-term potentiation (LTP) in a concentration-dependent manner. Dendritic production of cGMP as observed by Honda et al. (Honda et al., 2001) allows one to argue for a role of cGMP in the dendrites. However, the presence of PKG has not been shown at the postsynaptic side so far. We have shown that PKG II was highly present in the dendrites and co-localized with the postsynaptic marker PSD-95 in hippocampal neurons in culture.

III. Importance of N-myr in targeting of PKG II.

PKG II unlike PKG I is myristoylated on the N-terminal glycine. N-myr has been shown to be important for membrane targeting of PKG II (Vaandrager et al., 1996). The importance of N-myr in membrane targeting was shown by Western blotting of subcellular fractions and assaying protein kinase activity in HEK-293 and COS-1 cells. Recently it was demonstrated that the presynaptic cytomatrix protein Bassoon requires N-myr to be targeted to the synapse (Dresbach et al., 2003). The importance of N-myr in PKG II targeting in neurons is unknown. We chose to study the significance of N-myr on PKG II targeting by generating constructs containing different regions of the PKG II protein including G2A mutants of PKG II, which cannot be myristoylated.

We showed that N-myr is responsible for targeting FLwt PKG II GFP to the perinuclear region and membranes in COS-1 cells, HEK-293 cells and hippocampal neurons in culture. In COS-1 cells and HEK-293 cells, the FLwt and 5'wt PKG II are specifically targeted to the membrane and are concentrated at the distal ends of filopodia like structures, indicating possible interaction with actin binding proteins. VASP, a substrate of PKG (Waldmann et al., 1986), also accumulates at such focal adhesion points, where it interacts with actin binding machinery and regulates filopodia growth (Bear et al., 2002; Kwiatkowski et al., 2003; Laurent et al., 1999; Waldmann et al., 1986). In contrast both 5'PKG II G2A mutant and FL PKG II G2A mutant showed a diffuse distribution indicating that N-myr is important for proper subcellular targeting of PKG II. Recent studies using PKG I GFP proteins have shown that both FL PKG I GFP and the regulatory domain of PKG I tagged to GFP are diffusely localized in HEK cells (Browning et al., 2001). Browning et al. found that the PKG I

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regulatory domain, like PKG II regulatory domain in our study, is also found in dynamic regions of the plasma membrane. Browning et al. showed that when the PKG I catalytic domain was expressed without the regulatory domain, it accumulated in the nucleus. In this study, when the catalytic domain of PKG II was expressed without the regulatory domain, it did not translocate into the nucleus.

On the one hand, in hippocampal neurons in culture FLwt PKG II accumulated in the perinuclear area and had a punctate and membranous distribution in the dendritic processes. In the processes it co-localizes with the presynaptic marker synaptophysin and showed an accumulation at these probable synaptic sites. On the other hand the FL PKG II G2A mutant was much more diffusely distributed in the cell and did not accumulate at probable synaptic sites. This evidence supports the observation that PKG II is found concentrated in the synaptosomal membrane fractions and synaptic vesicle fractions in rat brain subcellular fractions. de Vente et al. also observed PKG II to be predominantly membrane associated in rat brain sections stained for PKG II (de Vente et al., 2001).

N-myr has been shown to be important for proper functioning of proteins. N-myr has been shown to be important for the stability of the PKA catalytic subunit (Carr et al., 1982) and for the transforming activity of $p60^{src}$ (Garber and Hanafusa, 1987). However, Vaandrager et al showed that myristoylation has no effect on either stability or activity of PKG II (Vaandrager et al., 1996). In the PKG II, N-myr appeared to be exclusively for targeting. Due to its intermediate hydrophobicity N-myr has been implicated in reversible membrane association (Taniguchi, 1999; Towler et al., 1988). Myristoylated alanine-rich PKC substrate

(MARCKS) is known to translocate between membrane and soluble fractions in a phosphorylation manner (Wu et al., 1982). As the intermediate hydrophobicity of N-myr is thought to be insufficient for membrane binding, it has been suggested that MARCKS binds membrane not only through its N-myr anchor but also through its PKC phosphorylation domain (Taniguchi, 1999). However, in the case of PKG II it has been suggested that N-myr alone would be sufficient as PKG II is a dimer and has two myr anchors per molecule of PKG II (Vaandrager et al., 1996). Although N-myr is thought to be an irreversible co-translational modification, demyristoylase activity has been demonstrated in the brain (Manenti et al., 1993; Manenti et al., 1994). Demyristoylation offers a novel way of changing the localization of proteins, but the significance of demyristoylation of specific proteins has not been well studied.

Subcellular localization of protein kinases and phosphatases provides a means to restrict where and when phosphorylation events occur. The importance of subcellular localization has already been demonstrated for PKG II. Membrane targeting of PKG II has been shown to be important for phosphorylation of the CFTR Cl⁻ channel. Although both PKG I and II can phosphorylate the CFTR Cl⁻ channel in vitro, only PKG II can phosphorylate the CFTR Cl⁻ channel in intact cells. This is due to its myristoylation and subcellular localization in the membrane (Lohmann et al., 1997; Vaandrager et al., 1998). It is also interesting to note that Wang et al. found a number of potential PKG substrates in peripheral membrane fractions from synaptosomes (Wang and Robinson, 1995). These are most likely to be substrates of PKG II as it is targeted to the membrane through myristoylation.

Subcellular localization of protein kinases can also occur through interaction with kinase anchoring proteins. A number of such proteins have been described for PKA. PKA has been shown to be targeted to cytoskeleton (MAP 2), endoplasmic reticulum (AKAP 100), Golgi (AKAP 85), mitochondria (AKAP 84), nucleus (AKAP 95), peroxisome (AKAP 220) and PSD (AKAP 79) (Coghlan et al., 1993; Coghlan et al., 1995; Hausken and Scott, 1996; Rubin, 1994). Such proteins have been described for PKC as well. They are called receptors for activated PKC (RACKs) and receptors for inactive PKC (RICKs) (Sim and Scott, 1999). PKA and PKG share a lot of structural similarities, both in the regulatory and catalytic subunits. Vo et al. used the regulatory domain of PKG II in a protein overlay assay and found a number of interacting proteins in the aorta, brain and intestine (Vo et al., 1998). They identified one ubiquitously expressed PKG II binding protein as myosin. However, Vaandrager et al. did not find any proteins in HEK cells that interacted with PKG II (Vaandrager et al., 1996), where PKG II is targeted to the membrane and possible focal adhesion points as we have observed in our study. Although the study of Vo et al. found interacting proteins from aorta tissue, PKG II, so far, has not been shown to be expressed in the aorta. In our study, we found that mutation of the N-terminal glycine, which renders PKG II non-myristolyated, leads to a diffuse localization of PKG II not only in HEK cells and COS cells but also in hippocampal neurons in culture. Dresbach et al. showed apart from myristoylation, a central region of bassoon is also required for targeting of bassoon to the synapse (Dresbach et al., 2003),. Our study indicates that N-myr of PKG II is an important factor for PKG II targeting. At the moment it is not known if N-myr alone is enough for PKG II targeting to the membrane and synaptic sites. Further deletion studies with the regulatory domain of PKG II have to be done to better understand the targeting of PKG II.

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IV. Probable role of PKG II in regulation of synaptogenesis..

One model for synapse formation predicts that active dendritic filopodia contact axons to induce presynaptic boutons, followed by a period of filopodial maturation into postsynaptic spines (Harris et al., 1992; Maletic-Savatic et al., 1999; Rao and Craig, 2000; Ziv and Smith, 1996). We have shown that overexpression of a possible dominant negative of PKG II in hippocampal neurons causes a dramatic increase in the number of filopodia at 10 div in hippocampal neurons and a similar increase in spines at 15 div hippocampal neurons, indicating that the increase in filopodia translates to spines. We have also shown that PKG II is expressed in the synaptosomal membrane fraction in both the adult and embryonic brain subcellular fractions. This implicates a role for PKG II in regulation of density of synaptic sites and hence a possible role in nervous system development and synaptic plasticity. Exactly how PKG II brings about this effect is still unclear. The most plausible explanation, based on the ability of the wt regulatory domain of PKG II to inhibit PKG activity, seems to be that inhibition of endogenous PKG II activity leads to an increase in the number of filopodia/spines. However, we did not find a decrease in density of filopodia and spines in FL wt PKG II overexpressing cells. It is likely that the PKG substrate is completely phosphorylated by the endogenous PKG II thus masking the effect of the recombinant protein. This indicates that PKG II probably plays a role in pruning of synapses. Consistent with this finding, NO has been shown to act as a "slow-down and search signal" in developing neurites and this effect was dependent on cGMP (Trimm and Rehder, 2004). NO has also been shown to cause collapse of growth cones and retraction of neurites (Ernst et al., 2000; Gallo et al., 2002; He et al., 2002). NO has also been shown to facilitate neurite outgrowth in PC12 cells in a cGMP dependent manner (Hindley et al., 1997; Rialas et al.,

2000). cGMP is also involved in inhibiting collagen-induced platelet aggregation, which requires dynamic actin reorganization followed by cell shape change {Aszodi, 1999 #347}. PKG mediates platelet aggregation through VASP {Aszodi, 1999 #347} and phosphorylation of VASP by PKA has been shown to reduce the ability of VASP to promote in vitro nucleation probably by reducing VASP binding to G-actin (Harbeck et al., 2000; Lambrechts et al., 2000; Walders-Harbeck et al., 2002). All this data is consistent with our finding that PKG II regulates filopodia/spine growth.

LTP , which is a model system for studying synaptic learning, produces an increase in the labeling of F-actin in the dendritic regions where LTP was induced but not in the control region (Fukazawa et al., 2003). Fukazawa et al also found that after LTP induction there was a persistent increase in the fraction of spines with high F-actin content and an increase in the diameter of spines. NO, cGMP and PKG are important for regulation of hippocampal neuron LTP (Arancio et al., 1995; Boulton et al., 1995; Lu et al., 1999; Son et al., 1998; Wu et al., 1998; Zhuo et al., 1994a). However, there is also evidence that indicates that this pathway is not involved in LTP (Schuman and Madison, 1994; Selig et al., 1996; Wu et al., 1998). Son et al. suggested that cGMP plays an important role in LTP under some conditions but not other situations (Son et al., 1998). Part of the discrepancy in the above studies may be due to the use of the commercially available PKG selective inhibitor KT-5823, which was recently shown to be not as selective to PKG as thought earlier (Bain et al., 2003; Burkhardt et al., 2000). It has been suggested by Son et al. that slight differences in protocols for induction of LTP could also be responsible for these different observations. Wu et al. suggested that the NO/cGMP/ PKG pathway is involved in LTD induction but not LTP induction in dentate

gyrus. They found that postsynaptic application of a PKG inhibitor inhibited zaprinast-induced LTD of EPSCs (Wu et al., 1998). Consistent with this finding Murphy and Bliss found that photolytically released NO (1-4.5 microM) from bath applied caged NO reduced the magnitude of long-term potentiation (LTP) in a concentration-dependent manner (Murphy and Bliss, 1999). Ko and Kelly, however, showed that extracellular application of the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) or postsynaptic co-injection of L-NAME with Ca(2+)/CaM blocked Ca(2+)/CaM-induced synaptic potentiation (Ko and Kelly, 1999). This indicates a postsynaptic role for PKG, which is consistent with our finding that PKG II was expressed on the postsynaptic side in hippocampal neurons in culture. Our finding that the dominant negative regulatory domain of PKG II causes an increase in filopodia and spines indicates that endogenous PKG II most likely plays a role in reducing the number of filopodia or spines, and thus is likely to play a role in LTD in hippocampal neurons. Our data suggest that for long-term plasticity, PKG II affects synaptic formation in the early stages, and may contribute to adult neuronal reorganization during learning.

Previous studies have shown that PKG I is present in hippocampal neurons in culture (Arancio et al., 2001; Kleppisch et al., 1999). We have shown that the wt PKG II regulatory domain can inhibit the activity of endogenous PKG I in HEK cells. It is still unclear if the effect seen is due to inhibition of PKG II activity or PKG I activity. The subcellular fractionation studies and immunocytochemical studies showing the presence of endogenous PKG II and not PKG I in synaptic sites indicate that the increase in filopodia/spines is most

likely due to the inhibition of PKG II. We have also shown that PKG II is present in higher concentrations than PKG I in hippocampal lysates.

It is not known so far as to what substrates of PKG might regulate filopodia/spine formation. The dynamic protrusion and retraction of filopodia is dependent on the regulation of actin binding and capping proteins (Rao et al., 2000). Ena/VASP proteins are a structurally conserved protein family found in vertebrates, invertebrates and *Dictyostelium* (Kwiatkowski et al., 2003). They have been shown to have an important function in cell motility (Anderson et al., 2003; Bear et al., 2000; Bear et al., 2002; Garcia Arguinzonis et al., 2002; Goh et al., 2002). VASP was initially identified as a PKA/PKG substrate in platelets (Halbrugge et al., 1990; Reinhard et al., 1992). Later on it was shown that all Ena/VASP members are substrates of PKA/PKG (Butt et al., 1994; Gertler et al., 1996; Lambrechts et al., 2000). One study showed phosphorylation of VASP decreases its affinity to F-actin (Harbeck et al., 2000) and another showed the opposite effect where phosphorylation showed an increase in affinity to F-actin (Laurent et al., 1999). VASP has been shown to promote actin polymerization and phosphorylation of VASP by PKA significantly reduces the ability of VASP to bind G-actin (Walders-Harbeck et al., 2002). Murine Ena (Mena) is enriched in filopodial tips of the neuronal growth cone and is required for proper axonal path-finding (Lanier et al., 1999). Based on the above data, we wanted to check if overexpression of human VASP would have an effect on the phenotype of hippocampal neurons. We found no differences in the number of filopodia/spines in the neurons transfected with human VASP when comapared to GFP overexpressing cells. There was also no difference in the density of spines/filopodia when VASP overexpressing cells were treated with 8Br-cGMP. It is unclear

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as to why we did not observe a morphological effect in VASP overexpressing neurons. It is possible that the overexpressed VASP is still fully phosphorylated by endogenous PKG II and does not enhance F-actin/filopodia formation. These data suggest that VASP is not involved in the PKG II pathway mediating increase in filopodia/spines, but not completely ruled out. It is possible that a yet unidentified Ena/VASP member might play a role in regulation of filopodia/spine formation in hippocampal neurons in culture. In our studies we used the human homologue of VASP and it is possible that it might not function in the same way as the rat homologue would.

Is it possible that the regulatory domain of PKG II can by itself reorganize the cytoskeleton and cause process outgrowth? It was recently reported that protein acylation confers localization to cholesterol and sphingolipid-enriched membranes (McCabe and Berthiaume, 2001). It has also been shown that presence of basic residues nearby the acylation motifs stabilize interactions with the negatively charged phospholipids present at the plasma membrane (Resh, 1999). Previous studies showed that alteration in the concentration of specific lipids alter membrane dynamics and fluidity. For example, addition of sphingomyelin or phosphatidyl ethanolamine analogs, lipids that expand the plasma membrane, increase the rate of cell spreading and lamellipodia extension and cause a decrease in membrane tension (Bershadsky and Futerman, 1994; Furuya et al., 1995; Harel and Futerman, 1993; Schwarz et al., 1995). It is possible that a similar mechanism is involved whereby the increased rate of addition of myristate to specific plasma membrane microdomains stimulates process outgrowth by a physical alteration of membrane tension. Alternatively, a change in membrane tension and expansion may stimulate the activation of

elements critical for recruitment and anchoring of specific proteins associated with filopodia extension at the plasma membrane. PKG II is myristoylated and also has basic residues in the vicinity of the myristoylation motif. However, it is not known if the dominant negative PKG II is targeted to lipid rafts. It is, however, unlikely that the regulatory domain alone might be inducing this growth effect, as the FLwt PKG II is myristoylated and has the entire regulatory domain, but does not induce filopodial or spine growth.

V. Conclusion

In this work we have identified a possible role for PKG II in regulation of synaptogenesis. Dendritic spine pathology has been associated with neurological disorders such as Alzheimer's disease and Creutzfeldt–Jakob disease and genetic disorders such as Down's and fragile-X syndromes (Fiala et al., 2002). In many brain regions, normal development involves an increase in synapses followed by pruning to mature levels (Huttenlocher and Dabholkar, 1997). Disruption of this developmental pruning may lead to increased spine density. For example, ovariectomy disrupts pruning in visual cortex (Munoz-Cueto et al., 1990). Similarly, an overabundance of dendritic spines in the reticular formation, vagal nuclei and ventrolateral medulla in infants dying from sudden infant death syndrome is thought to be a failure of developmental synapse elimination, and appears to be involved in the defective cardiorespiratory regulation (O'Kusky and Norman, 1994; Quattrochi et al., 1985; Takashima and Becker, 1985; Takashima et al., 1985). Defective pruning has also been shown to be responsible for increased spine density seen in phenylketonuria (Lacey, 1985) and fragile-X syndrome (Irwin et al., 2001). Identifying and studying the molecules

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involved in regulation of filopodia/spine formation will contribute to our understanding of not only the normal dendritic spine function, but also the pathogenic aspects of central nervous system.

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