Research Report and Proposals

In Partial Fulfillment of the Requirements

for the Degree of Master of Science

Thomas Kingsley Brown

Department of Chemistry

California Institute of Technology

April 28, 1989

Research Report:

Structure and Function of Neuronal Postsynaptic Densities of the Central Nervous System

Research Proposals:

I. Investigation of Placebo-induced Analgesia by PET Scanning of Opiate Receptors

II. Investigations of the Binding Properties of a Possible PSD-binding Domain in the Alpha Subunit of the Type II CaM Kinase

Table of Contents

Introduction

The postsynaptic density (PSD), as visualized by electron microscopy, is a disc-shaped, densely staining protinaceous structure which is firmly attached to and partially co-extensive with the postsynaptic plasma membrane of central nervous system dendritic spines. One side of the PSD thus faces the area between the neurons at the site of synaptic transmission (the "synaptic cleft") and the other side faces the dendritic cytoplasm. One feature of interest of the PSD is that since it is so closely apposed to the site of neurotransmitter release, it is highly likely to contain proteins which either function to maintain receptors near the synaptic locus, or which are receptors themselves. Additionally, it may contain proteins whose morphology is dependent on the frequency of presynaptic neuronal firing, and whose effect on dendritic shape may thus be involved in some aspects of long-term potentiation.[1] Since the early 1970's, with the advent and development of a procedure which allowed the isolation of essentially pure PSD fractions from brain homogenates, the PSD has become directly accessible to biochemical study. The isolation procedure was initially documented by deRobertis and coworkers[2] and later modified by several groups.[3-5] It employs differential centrifugation to isolate synaptosomes, followed by detergent extraction to remove membranous material. Biochemical and electron microscope studies of PSD fractions and brain sections have revealed the identity of a handful of component PSD proteins[6,7] and have produced some information about PSD substructure. However, much remains to be determined. Few of the results yielded by electron microscopy studies are free from ambiguity, and current con-

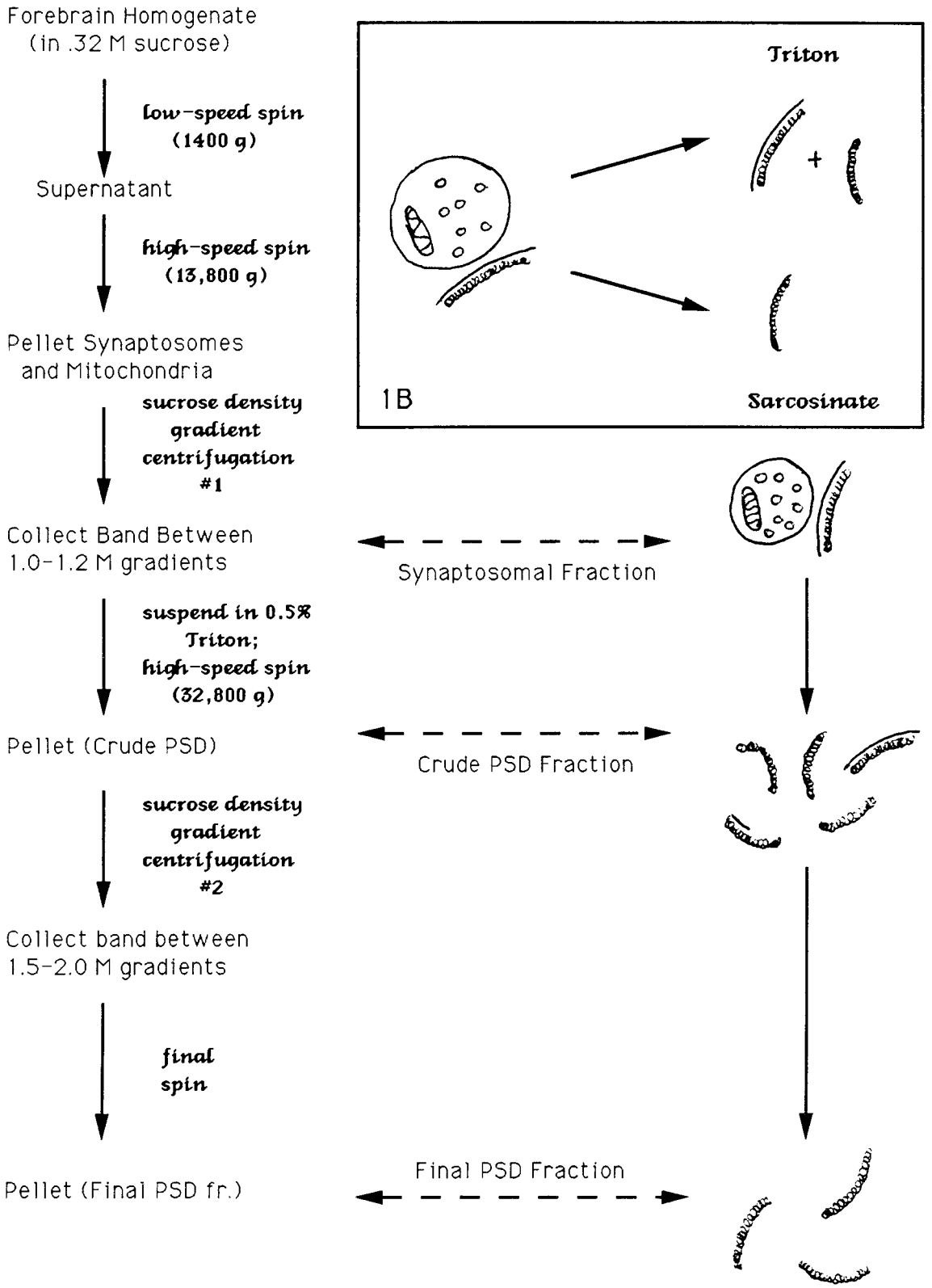
cepts of PSD structure and function are largely interpretations based on scant concrete information. For example, Andrew Matus observes the PSD to be composed of a planar array of small dense-staining subunits[8] often surrounding "one or more island(s) of fine granular material."[9] He proposes that "the granular enclosures represent active sites which are kept in place by the surrounding subunits."[10] Another researcher, Dennis Landis, has observed a fine filamentous meshwork with associated globular attachments of varying size, located on the cytoplasmic face of the PSD.[11] He interprets these globular structures to be cytoplasmic proteins attached to PSD filaments, and attributes differences between these structures amongst different PSD's to possible differences in constituent proteins of the neurons.[12] However, Landis also notes that what is seen in the electron micrographs varies with the methods of fixation and staining, and is probably affected by variations of technique within methods.[13] It is apparent, then, that much additional work is required in order to establish a clear understanding of PSD function. The research project I will now describe is designed to help elucidate PSD structure and to get a better understanding of how this structure imparts PSD activities. The general strategy of the project is to use newly available techniques to study selected individual proteins of the PSD, and to deduce their roles in PSD function. This will involve first isolating a particular protein and producing proteolytic fragments; we will then obtain the amino acid sequences of several of these fragments. This information will be used to generate antibodies and to select and sequence cDNA clones. At this point we will address questions about the protein's function and local-

ization in the brain.

Preparation of PSD Fractions

Direct biochemical analysis of PSD's was made possible by the development, in the 1970's, of a procedure to isolate PSD fractions from brain homogenates.[2-5] An example of a modification of this procedure is shown diagramatically in figure 1A.[14] The first few steps use differential centrifugation to separate out what is called the "crude mitochondrial fraction" which contains mitochondria as well as synaptosomes. The crude mitochondrial fraction is then subjected to the first of two sucrose density gradient centrifugations. This serves to separate mitochondria and synaptosomes. The band between the 1.0 and 1.2 molar sucrose gradients contains the "synaptosomal fraction." This fraction is then suspended in a Trition X-100 solution (0.5%) which preferentially solubilizes the membrane lipids and proteins, leaving the "crude PSD fraction" which contains predominantly PSD's as well as a small amount of contaminating membrane fragments and some semi-intact synaptic junctions. A more refined PSD fraction is obtained by the crude PSD fraction to a second sucrose gradient centrifugation, collecting the appropriate band, and suspending this in another Triton solution to extract most of the remaining membrane components. An ultracentrifuge spin of the Triton solution produces the final PSD fraction, which is a clear, sticky pellet containing about 0.2-0.3 mg of protein. In later preparations, we ended the procedure after collecting the "crude PSD fraction" (obtained after the first Triton extraction) since we discovered that

Fig. 1

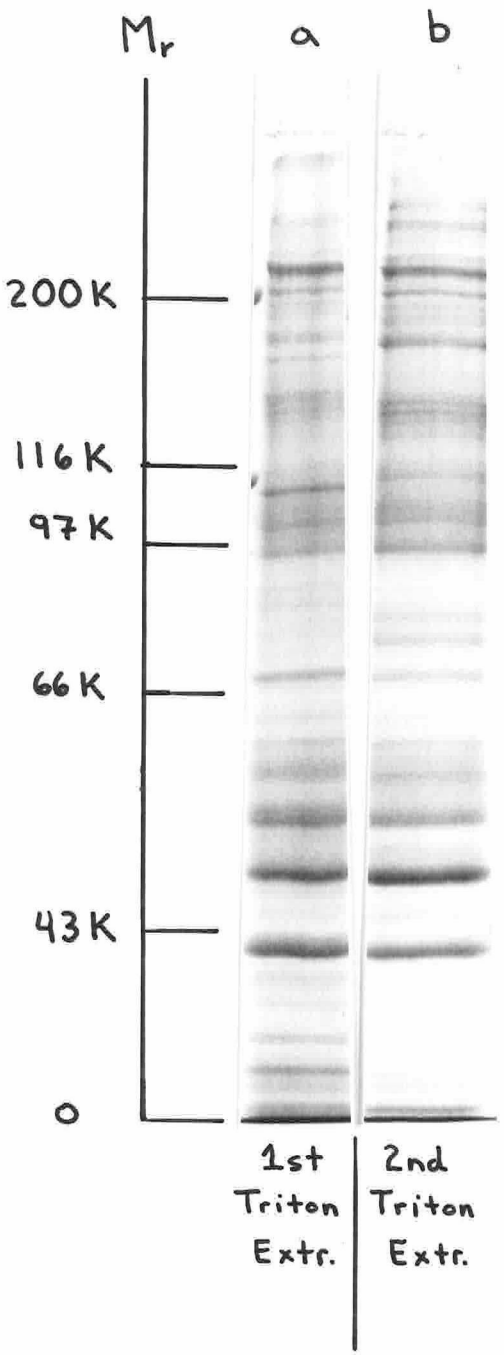

Forebrain Homogenate
(in .32 M sucrose)
low-speed spin
(1400 g)
Supernatant
high-speed spin
(13,800 g)
Pellet Synaptosomes
and Mitochondria
sucrose density
gradient
centrifugation
#1
Collect Band Between
1.0-1.2 M gradients
suspend in 0.5%
Triton;
high-speed spin
(32,800 g)
Pellet (Crude PSD)
sucrose density
gradient
centrifugation
#2
Collect band between
1.5-2.0 M gradients
final
spin
Pellet (Final PSD fr.)
Triton
+
1B
Sarcosinate
Synaptosomal Fraction
Crude PSD Fraction
Final PSD Fraction

this fraction easily yielded 15-20 times as much protein (about 4-5 mg) and differed little from the "final PSD fraction" in respect to the assortment of proteins present, as determined by comparison using gel electrophoresis of the fractions (see figure 2).

Another variation of this preparation procedure involves using n-lauryl sarcosinate rather than Triton as a detergent (see figure 1B).[15] It is found that the sarcosinate procedure removes a greater percentage of the membranous component from the synaptosomal fraction than does Triton,[16] thus producing a simpler PSD fraction. However, one disadvantage of the sarcosinate procedure, and the main reason why we have decided to use the Triton procedure instead, is that the protein yield is much lower when sarcosinate is used than if Triton is used.[17] Also, while sarcosinate removes a greater proportion of the membrane contaminants, it may also tend to remove some of the less tightly bound PSD proteins[18] so that it is possible that the Triton extractions may actually produce a PSD fraction which is more accurately representative of the true PSD components.

The issue of what the PSD fractions actually represent is one of considerable uncertainty. It is possible that a particular protein's presence in the PSD fraction may simply be an artifact of the isolation procedures, rather than a certain indication that the protein is present in the PSD *in vivo*. Discepancies between the components of the PSD fraction and the actual PSD could conceivably have multiple causes. For instance, a protein which resides in the neuronal plasma membrane nearby the PSD (but not in the part of the membrane which is co-extensive with the

FIG. 2



PSD) may partition with the PSD fraction if the step involving the suspension of the synaptosomal fraction in a detergent solution is not completely efficient in separating the PSD from the non-PSD membrane components. Problems can also arise because of the physical nature of the PSD fraction. The fraction is a rather adhesive conglomerate, and may tend to aggregate with non-PSD proteins which are present in the starting brain homogenate. In fact, it has been demonstrated that radiolabelled glial fibrillary protein (GFA), when added to brain homogenate at the beginning of a PSD isolation procedure, can be detected in the final PSD fraction.[19] In consideration of these problems, an important goal of this project, in addition to the elucidation of PSD structure, will be an improved definition of the degree of usefulness of the PSD preparation procedures for investigations of PSD structure and function.

Selection of Specific Proteins

In order to study individual proteins of the PSD fraction, we must first separate them from one another on a polyacrylamide gel by electrophoresis (for example, see fig. 2). There are over 30 distinct protein bands in the fraction; however, fewer than ten of these proteins have been identified and shown experimentally to exist in the PSD (see table 1). While most of these known proteins of relatively low molecular weights -- below 65 KD -- it is apparent from the PAGE gels that the majority of the proteins in the PSD fraction are of relatively high molecular weights -- above 90 KD. Also, three of the known proteins (actin,

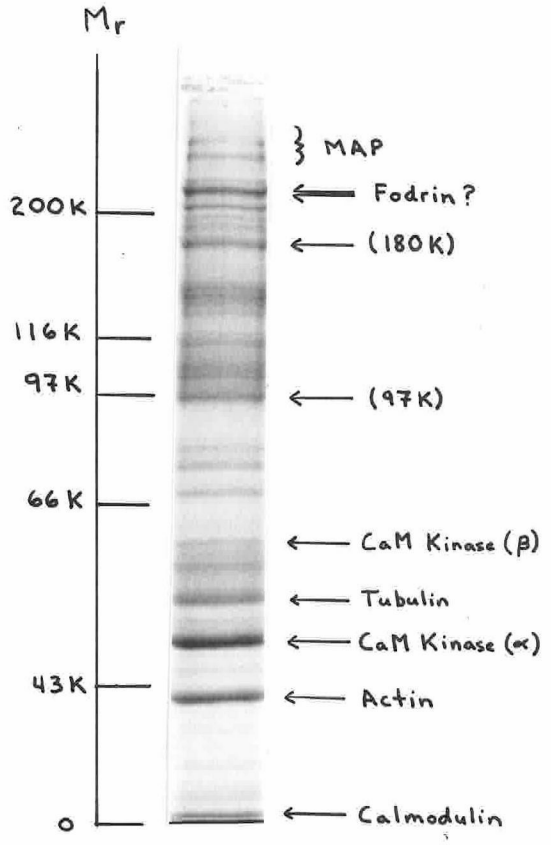
Table 1

Proteins Thought To Be In PSD's

	M.W. (KDa)
CaM Kinase	50 & 60
Actin	46
Tubulin	55
Fodrin	230 & 235
Calmodulin	18
Microtubule Assoc. Pro.	~ 300
Glutamate Rec.*	unknown
GABA Rec.*	unknown

*see legend

FIG. 3



tubulin, and fodrin) probably function mainly to maintain and regulate synaptic junction morphology, and it seems unlikely that the remainder of the known proteins represents a large proportion of those which mediate all PSD functions. There should be many unidentified proteins in the PSD fraction, then, which are vital for the PSD's activity and which we would therefore have an interest in studying. Because there are many distinct proteins in the PSD fraction, and since we wish to focus on only a few at a time, we have established criteria by which to select the most appropriate ones. As a result of screening the fraction for proteins having the desired attributes, we have decided to initially investigate two proteins, one at 95 KD and one at 180 KD (see fig. 3).

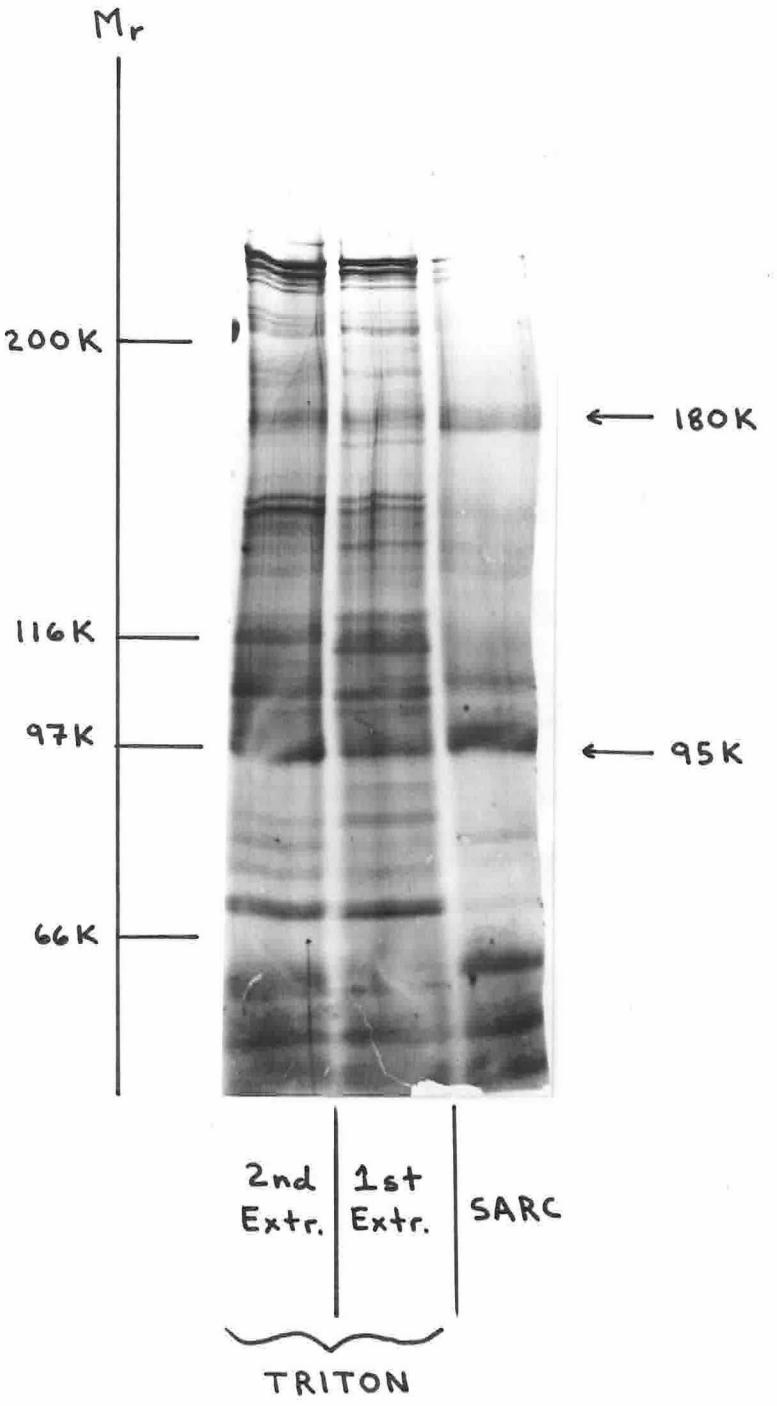
There are a few criteria which must be met by a protein for it to be a candidate for our sequencing efforts. First, it is necessary that the protein be abundant enough in the PSD fraction so that we can collect, without too much preparative effort, the minimum amount of the protein which we are able to sequence using our techniques. One way to estimate the abundance of a protein is to compare its intensity of staining on a gel with those of standards of known abundance. Also, a protein's relative abundance can be judged by comparing its staining intensity to the intensities of the other proteins in the PSD fraction, and since we know the total amount of protein present in the fraction we can calculate an estimate of the absolute abundance. In order to obtain a portion of the amino acid sequence from a protein, we will need a minimum of approximately one nanomole of the protein. We have estimated that we will require less than one week to collect this much of the

180 KD protein, and only a few days in the case of the 95KD protein. Most of the other high molecular weight proteins are not as abundant in the fractions as are these two, however, and gathering enough material to study them would involve considerably more effort. There are many other proteins besides the ones at 95 KD and 180 KD which are within our practical reach for later investigation, but one reason we have chosen to initially study these two is that they offer the advantage of relative experimental expediency.

Another criterion that the proteins we select should meet gives us an indication of the protein's probability of being located in the PSD. This is important, since, as mentioned in the "PSD preparation" section of this paper, a protein's presence in the PSD fraction does not necessarily indicate that it is a component of the PSD *in vivo*. We would like to overcome this difficulty, in order to reach a greater degree of confidence that the protein(s) we choose to sequence have a high probability of being located in the PSD. One way to do this is to compare the constituents of PSD fractions prepared using a variety of preparation procedures. As mentioned earlier in this paper, the composition of the fractions produced varies considerably depending on which detergent (Triton or sarcosinate) is used, and there are relatively minor qualitative differences in the fractions produced using Triton, depending on whether the fraction is collected after the first or second detergent extraction ("crude PSD" versus "final PSD" fractions -- see fig. 1). A protein's abundance in a fraction made by one procedure relative to its abundance in a fraction made by another procedure can give us an indication of the likelihood of that protein

being present in the PSD *in vivo*. Figure 4 shows a comparison of patterns created by gel electrophoresis of protein fractions produced by three different procedures. Interpreting the differences between the two lanes of Triton fractions is fairly straightforward. A protein which is diminished in relative abundance in going from the "crude PSD fraction" to the "final PSD fraction" may be a non-PSD membrane protein or other non-PSD contaminant, since the PSD proteins are thought to be tightly bound to one another, and would resist solubilization in the detergent solution more strongly than would the non-PSD proteins. By the same reasoning, a protein which is relatively enhanced in the "final PSD fraction" as compared to the "crude PSD fraction" is more likely than others to be an actual PSD component. Similar comparisons can be made between the fractions prepared using Triton and the one prepared using sarcosinate (with proteins in the sarcosinate fraction having a higher likelihood of being actual PSD components compared to proteins which are present in the Triton fractions but not in the sarcosinate fraction). Since we would like to study PSD proteins, we have used the comparisons of the various fractions by selecting the proteins for our investigations from amongst those which are relatively enhanced (or at least not relatively diminished) in the more stringent preparation procedures. The two proteins we have chosen to initially study (95 KD and 180 KD) both obviously fit this selection criterion, as can be seen in figure 4. Especially notable is the sarcosinate fraction, in which most of the proteins with M_r's of above 90 KD have been largely eliminated, leaving the 95 KD and 180 KD proteins as the two major high molecular weight bands.

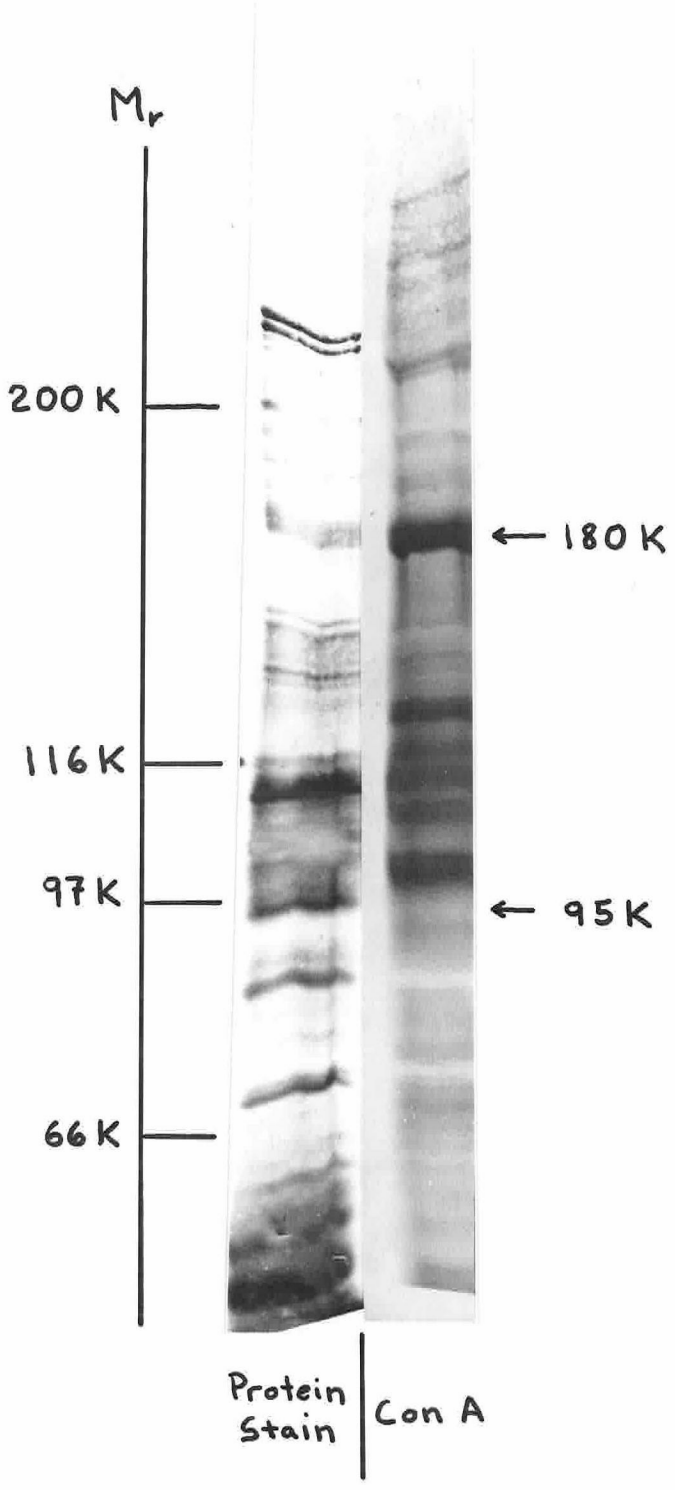
FIG. 4



In addition to the criteria which a protein must necessarily meet in order to be selected for initial investigation, there are also a few qualities which can enhance our interest in some proteins relative to the others which meet the aforementioned minimum requirements.

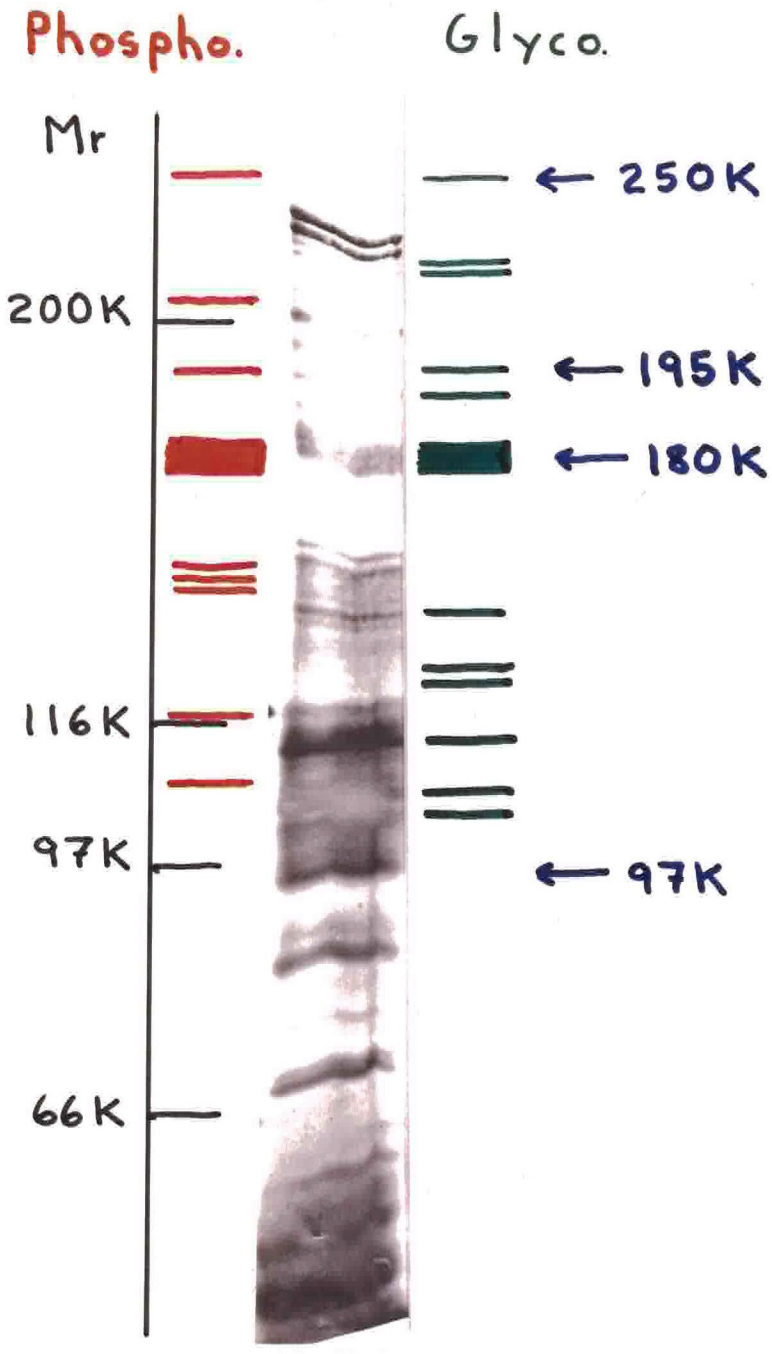
It can be fairly easily determined which proteins in the PSD fraction are glycosylated and which are not. The procedure for this determination takes advantage of the affinity of the glycoproteins for a lectin, "concanavalin A" (hereafter referred to in this paper as "Con A").[20] If a particular protein is a glycoprotein, then it is almost certainly an integral membrane protein. If it is also in fact in the PSD, then it must be located within the postsynaptic plasma membrane, where it would be perfectly situated to act as a receptor protein, for example, or at least to mediate some other function related to the communication between neurons. To determine which proteins in the PSD fraction are glycoproteins, a PAGE gel of the fraction is transferred to nitrocellulose, and this blot is then washed in a solution of Con A, which binds to the glycoproteins. A control blot is washed in a solution containing Con A and alpha-D-methyl mannopyranoside. The blots are then washed in a solution of horseradish peroxidase (HRP). Since Con A is multivalent, and since HRP is a glycoprotein, HRP binds to the Con A and a conglomerate of PSD fraction glycoprotein plus Con A plus HRP is formed, and this conglomerate is then visualized by the reaction of HRP with 3,3-diaminobenzadine. Figure 5 shows the results of such an experiment. We have identified several glycoproteins by this technique, most notably the protein at 180 KD which we have chosen to study.

FIG. 5



Another experiment which can be performed to distinguish proteins in the PSD fraction which are of particular interest is to determine which ones are substrates of the Type II Ca^{2+}/calmodulin dependent protein kinase (CaM kinase), which is currently being investigated by members of the Mary Kennedy research group at Caltech. In this procedure, an aliqout of the PSD fraction is incubated with CaM kinase, calmodulin, Ca^{2+}, and (^{32}P)ATP. The mixture is then separated by PAGE, and the phosphorylated proteins are visualized by autoradiography. Proteins phosphorylated by the kinase *in vitro* would presumably be phosphorylated *in vivo* as well, indicating that they are regulated by the neuron for some reason (in the case of the PSD proteins, this reason would probably be related to some aspect of synaptic transmission). Figure 6 displays the results of the efforts to detect the phosphoproteins of the PSD fraction, alongside the results of the glycoprotein identification experiments. There are over ten proteins in either category, but only three which are both glycoproteins and phosphoproteins (at 180 KD, 195 KD, and 250 KD). The proteins at 195 KD and 250 KD are both minor bands, however, and would thus be more difficult to study than would be the 180 KD protein, which is more abundant, and which in fact fits each of the criteria by which we have selected proteins for sequencing. The 95 KD protein, which we also plan to sequence, is neither a glycoprotein or a phosphoprotein, but was chosen for study mainly because of its relative abundance and because it becomes more predominant relative to the other proteins in the more stringent PSD preparation procedures.

FIG. 6



Planned Research Strategy

The first phase of our research investigating individual PSD proteins, which has already been described here, is the selection and isolation of the proteins which best fit our interests and research techniques.

The first step in the sequencing of a selected protein is an enzymatic digestion of the protein. We will use a method recently developed at Caltech by Aebersold and Kent *et al.*[21] In this method, the PSD fraction is separated by PAGE and then transferred to nitrocellulose. The proteins are then stained reversibly, and the appropriate band is removed and subjected to a trypsin digest while still in the nitrocellulose matrix. This method is more efficient than digestions performed directly following PAGE separation, as less protein is needed in order to generate large enough quantities of tryptic fragments for gas-phase sequencing. After enzymatic digestion of the protein, the tryptic fragments will be separated by HPLC, and several fragments sequenced. These fragments will then be used to initiate two different projects simultaneously. One project will be the determination of the protein's amino acid sequence, and the other will be aimed at examining the protein's localization and distribution in the brain. In the sequencing project, knowledge of the tryptic fragment sequences will be used to generate chemically-synthesized oligonucleotides which code for portions of the protein. The oligonucleotides will then be radiolabelled and used as probes to select appropriate complementary DNA from a rat brain cDNA library, which will be immobilized on a solid support. The protein's amino acid sequence can then be inferred after the cDNA sequence is deter-

mined. The knowledge of the protein's amino acid sequence may allow us to infer possible functional roles of the protein. We will search for sequence homologies between our selected protein and complements of currently known proteins in computer databanks such as the "National Biological Research Foundation Protein Identification Resource (NBRF-PIR)" and the "NEWAT" database.[22] We must set the algorithms which determine what constitutes sufficient homology, and the computer will search for the homologies.

For the project to determine the protein's localization, we will use the tryptic fragments to create antibodies, which will be used in immunohistochemical studies with rat brain slices. The peptides will be attached to hemocyanin, which is non-antigenic, and injected into rabbits to produce peptide antisera. The specificity of the anibodies will be checked through Western blots against protein from whole rat brain and rat PSD's. Brain slices will be immersed in solutions containing the antibodies, and the location of the antibodies can then be determined by incubation of the slices with gold-labelled protein A which can be visualized by electron microscopy.

The PSD fraction protein's localization will be interesting regardless of whether or not it is confined to the PSD's. If we find that the protein is, as we would expect, located within PSD's, then it will be of interest to know, for instance, whether it is present in all PSD's, or if perhaps its presence varies according to brain region or according to factors particular to only certain types of PSD's. According to current common beliefs of researchers of PSD structure and function,

the proteins of the PSD fractions constitute a fair representation of the PSD components *in vivo*. Of the proteins present in the PSD fraction, the ones that we will initially study (at 95 KD and 180 KD) are two of the most likely to be located in the PSD (by virtue of comparisons of various preparation techniques, as detailed earlier in this paper). Therefore, finding that either or both of these proteins are not located in the PSD would justify a re-evaluation of common assumptions about the PSD fractions and the nature of the PSD itself.

Figure legends

Fig. 1: Adapted from Carlin *et al* (1980)[14]; (A): Schemata for isolation procedure of postsynaptic densities (see text for explanation). The right side of the figure lists the major steps of the procedure, and the left side shows representations of the components present in the corresponding step to the right. (B): Diagram representing differences between PSD fractions prepared using Triton and n-lauryl sarcosinate detergents.

Fig. 2: Comparison of 8% PAGE gels of PSD fractions prepared using Triton detergent. Lane "a", "crude PSD fraction" and lane "b", "final PSD fraction"; 0.04 mg total protein in each lane.

Fig. 3: 8% PAGE gel of PSD fraction proteins (0.04 mg) with arrows denoting positions of known proteins in the fraction.

Fig. 4: 6% PAGE gel gel transferred to nitrocellulose comparing PSD fractions prepared by different methods; left lane, "final PSD fraction" prepared using Triton; middle lane, "crude PSD fraction" prepared using Triton; right lane, fraction prepared using sarcosinate. Each lane contains about 0.01 mg protein.

Fig. 5: 6% PAGE gel of PSD fraction transferred to nitrocellulose; left lane, 0.015 mg protein stained with a general protein stain; right lane, 0.08 mg protein transferred to nitrocellulose and subjected to Con A/HRP treatment to label glycoproteins; not shown, control lane in which 0.08 mg of protein was incubated in a solution of Con A + alpha-d-methly mannopyranoside and then HRP -- essentially nothing was labelled in this lane.

Fig. 6: 6% PAGE gel transferred to nitrocellulose, with red marks on the left indicating positions of proteins which are phosphorylation substrates of Type II CaM kinase, and green marks on the right indicating positions of glycoproteins identified by Con A/HRP procedure. Arrows indicate proteins which are both glycoproteins and phosphoproteins.

Table 1: List of proteins thought to exist in the PSD *in vivo* (from Matus, 1981)[6]; presence of glutamate receptor and GABA receptor proteins in PSD's has not been confirmed -- their presence is indicated, however, by studies demonstrating the binding of glutamate and GABA to PSD fractions.

References

1.) Siekevitz, P. (1985) The Postsynaptic Density: A possible role in long-lasting effects in the central nervous system. *Proc. Natl. Acad. Sci.* **82**, 3494-98

2.) deRobertis, E., Azcurra, J.M., and Fiszer, S. (1967) Ultrastructure and cholinergic binding capacity of junctional complexes isolated from rat brain. *Brain Res.* **5**, 45

3.) Davis, G., and Bloom, F.E. (1970) Proteins of synaptic junctional complexes. *J. Cell Biol.* **47** (2, Pt. 2), 46a (Abstract)

4.) Cotman, C.W., and Flansburg, D. (1971) An analytical micro-method for electron microscopic study of the composition and sedimentation properties of sub-cellular fractions. *Brain Res.* **22**, 152

5.) Cohen, R.S., Blomberg, F., Berzins, K., and Siekevitz, P. (1977) The structure of postsynaptic densities isolated from dog cerebral cortex, Part II. *J. Cell Biol.* **74**, 181-203

6.) Matus, A. (1981) The Postsynaptic Density (Review). *TINS*, 51-53

7.) Shaw, G. (1984) Postsynaptic densities clarified? *Nature* **308**, 496

8.) Matus, A., and Walters, B. (1975) Ultrastructure of the synaptic junctional lattice isolated from mammalian brain. *J. Neurocyt.* **4**, 369-375

9.) Matus, A. (1981)

10.) *ibid*

11.) Landis, D.M.D., Weinstein, L.A., and Reese, T.S. (1987) Subtructure in the postsynaptic density of purkinjie cell dendritic spines revealed by rapid freezing and etching. *Synapse* **1**, 552-558

12.) *ibid*

13.) *ibid*

14.) Carlin, R.K., Grab, D.J., Cohen, R.S., and Siekevitz, P. (1980) Isolation and characterization of postsynaptic densities from different brain regions; enrichment of different types of postsynaptic densities. *J. Cell Biol.* **86**, 831-843

15.) Matus, A. and Taff-Jones, D.H. (1978) Morphology and molecular composition of isolated postsynaptic junctional structures. *Proc. R. Soc. London B.* **203**, 135-151

16.) *ibid*

17.) *ibid*

18.) *ibid*

19.) Matus, A., Pehling, G., Ackerman, M., and Hauser, K. (1984) Surface antigens of brain synapses -- identification of minor brain proteins using polyclonal antisera. *J. Cell Biol.* **98**, 237-248

20.) Kelley, P.T., and Cotman, C.W. (1977) Identification of glycoproteins and proteins at synapses in the central nervous system. *J. Biol. Chem.* **252**, 786-793

21.) Aebersold, R.H., Leavitt, J., Saavedra, R.A., Hood, L.E., and Kent, S.B.H. (1987) Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after *in situ* protease digestion on nitrocellulose. *Proc. Natl. Acad. Sci. USA* **84**, 6970-6974

22.) Bishop, M.J., and Rawlings, C.J., editors (1987) *Nucleic Acid and Protein Sequence Analysis: A Practical Approach.* IRL Press (Oxford)

Proposed Investigation of Placebo-induced Analgesia

by PET Scanning of Opiate Receptors

Results from many investigations throughout the 1970's indicated that endogenous opiods were likely to have a role in physiologically-mediated analgesia.[1] The first studies showing the exitstence of a possible endogenous pain-control system were those in which stimulation of certain areas of the brain led to analgesia in rats.[2] It was later found that analgesia of this type, called "stimulation-produced analgesia" (SPA) was reversible by the morphine antagonist naloxone[3] and cross-tolerant with morphine.[4] SPA in man was also shown to be accompanied by an increase in cerebro-spinal fluid levels of an enkephalin-like material.[5] From this information it was inferred that an endogenous opioid system might be responsible for SPA. However, since the analgesia in these experiments was produced in a physiologically abnormal manner (i.e., external stimulation of the brain) the relevence of the results to normal endogenous activities was uncertain.[6] Later experiments were designed to create conditions in which endegenous pain control mechanisms would be activated in a more normal manner.[7] The usual strategy of these studies was to show that analgesia could be produced in rats by subjecting them to stressful or painful stimuli, and then to show that this analgesia could be reversed by naloxone.[8] A similar type of investigation showed that electrical footshock induced analgesia in rats; the connection to opioid systems was demonstrated by autoradiography of brain slices which showed that the footshock reduced the binding of a radiolabelled opiod agonist.[9] Experiments such as this provided considerable evidence that endogenous opioid release and analgesia were linked; however, they could not conclusively prove a direct cause and effect relationship between the two.[10]

Before the 1980's, several endogenous peptides were known which contained the opioid core of Tyr-Gly-Gly-Phe-Met (or -Leu), but their relationships were not known.[11]This was changed with the discovery that all opioids belonged to any one of three opioid families, each with its own distinct genetic origin.[12] It has also been discovered that distinct opioid receptor types exist in the brain. The idea of distinct

types was first documented in 1976 by Martin and co-workers,[13] but not until very recently has a fairly common consensus been reached that there are three types, known as the mu-, kappa-, and delta-receptor types.[14] Each receptor type has its own distinct and heterogenous distribution in the brain. The mu-receptor type, for instance, is particularly prevalent in the thalamus, hypothalamus, parietal cortex, and periaqueductal gray matter.[15] It appears that the puzzle of which opiods bind to which receptors is far from simple; it is not the case, at least, that each family of opioids binds to only one type of receptor. Some of the endogenous ligands, as well as other agonists and antagonists, have been found to show a distinct preference for one type of receptor over the others, while some ligands seem to bind to two or three types with approximately equal affinity.[16] Morphine has been shown to display a large preference for the mu-receptor type -- hence the origin of the name "mu" (for morphine) for that receptor. Since morphine and its antagonist, naloxone, seem to affect endogenous pain control, the mu receptor has been implicated as being involved in pain attenuation.

A recently developed technique has emerged which may allow the relationship of mu receptors to analgesia to be more thoroughly tested. Positron Emission Tomography (PET), a method which has been used to measure cerebral blood flow and regional utilization of oxygen and glucose, has been adapted for use in monitoring transmitter release and receptor occupation levels.[17] PET is a method for measuring the distribution of positron-emitting isotopes in the brain and other body areas. Biologically interesting isotopes which are postitron emitters include ^{15}O, with a half-life of 2 minutes; ^{13}N (10 min); ^{11}C (20 min); and ^{18}F (110 min). Examples of their uses include [^{15}O]O_2, used for measuring oxygen metabolism, and [^{18}F]fluorodeoxy-glucose for measuring glucose use. ^{11}C and ^{18}F have been used in the creation of a few dozen positron-emitting drug analogs for use in *in vivo* pharmacology studies. Opiate receptors were first imaged using [^{11}C]carfentanyl, which has an affinity for opiate

receptors which is about 8000 times as strong as that of morphine, and which is about 90- to 250-fold less potent at delta and kappa receptors, respectively, than at mu receptors *in vivo*.[18] In order for a ligand to be useful in imaging receptors, it must achieve a high ratio of specific:nonspecific binding in the brain at some time after injection.[19] Nonspecific, or background, binding can be estimated for opiate agonists and antagonists by measurement of the ligand's binding in the cerebellum, which is essentially free of opiate receptors; specific binding can be assumed to be measurable by subtracting cerebellar concentrations from concentrations in other brain regions.[20] Changes in receptor occupancy can also be measured, as demonstrated by a study in which the binding of [^{11}C]carfentanyl to receptors in the human CNS was 90% displaceable by naloxone (a highly specific antagonist of mu receptors) in a dose-related manner.[21] Besides displaying the specificity of carfentanyl for mu receptors, this also suggests that it should be possible to use [^{11}C] carfentanyl to monitor changes in binding by endogenous opioid ligands, which would be a useful technique in an experiment designed to test, in humans, the possibility of a causal link between mu-specific ligand release and endogenous analgesia.

Another important element in such an experiment would be a scheme for activating the body's pain control systems; obviously, the use of highly painful stimuli in such a capacity would be considered unethical within the standards of the modern scientific community. A nonviolent alternative towards this purpose may exist in a medical technique which has demonstrated a morphine-like ability to attenuate pain, and which might possibly produce its effects by mobilizing the body's native analgesic potential.

This medical technique employs what is known as the "placebo effect", which is demonstrated by physiological changes brought about by a patient's expectations of the effect of a "drug" which actually has no pharmacologically active properties of its own. In some cases, a patient's physiological response to expectations of a placebo's action

can actually reverse the pharmacological action of a drug.[22] In regards to pain relief, placebos have been shown to be almost as effective as morphine in reducing post-operative pain.[23] It has also been demonstrated that placebos can create other morphine-like effects; in one experiment, morphine addicts were injected with a placebo (a saline solution) in place of the actual drug, and did not show any morphine withdrawal effects until the placebo injections were discontinued.[24] It seems quite possible that this effect was produced by the stimulation of morphine receptors. This would be an indication that stimulation of opiate receptors can be induced by a person's expectation of a physiological effect from a placebo. It is therefore possible that a subject's expectations of analgesia could cause activation of endogenous pain control systems, and may help to provide a way to address the issue of possible mu receptor involvement in endogenous analgesia.

In this research proposal I will outline an experiment designed to test the hypothesis that endogenously-produced analgesia is caused, in part, by the release of mu-receptor-specific opioid ligands. In the experiment, placebos purported to provide pain relief will be administered to chronic pain sufferers. Changes in endogenous mu-specific ligand concentrations will be monitored by the presence of the mu-agonist [^{11}C]carfentanyl. If the placebo does in fact lead to the release of mu-specific opioids, there should be a decrease in mu-specific binding of the radiolabelled agonist. This decrease should be especially notable in the brain areas known to be most highly enriched in mu receptors.

Experimental Setting

Subjects for the experiments will be chosen from amongst chronic pain sufferers on a voluntary basis. Advertisements for the tests will mention the need for subjects in order to test a "powerful new method for stimulating the body's own ability to reduce pain" and will discuss the requirement of exposure of the participants to low levels of radiation, as well as a short period of abstinence from the subject's usual pain

relief medicine (this will allow time for the drugs to clear from the brain so that they will not interfere with the tests).

Briefing of the subjects before the test will mention that "the body is equipped with its own powerful system for reducing pain, which we believe operates at far below its potential efficiency in most persons. This medication you are about to take is an experimental drug designed to enhance this system's efficiency by stimulating the release of the body's own pain-relief drugs. It has been shown to act quickly and powerfully in many patients." Of course, this monologue would be aimed at building the subject's expectancy of pain relief and increasing confidence in the body's ability to provide such pain relief (an element shown to often be an important factor in a patient's recovery from disease).[25] The subject will also be told that "the PET scanning is to be used to monitor the position of the synthetic drug, so that we will know when the drug has entered the brain, and also what areas of the brain are being affected." The subject will be given an injection of [^{11}C]carfentanyl, the presence of which will be monitored in the brain to record pre-placebo levels of binding, and time will be taken to allow for optimal specific:nonspecific ratios of binding while the placebo is ingested. The placebo will be administered, and [^{11}C] levels monitored continually.

Following the experiment, the subject will be asked to fill out a questionnaire concerning the subject's own perception of the magnitude of the pain relief supplied by the drug. This will allow the test of a correlation between changes in receptor occupancy (and hence the release of mu-specific ligands) with the apparent level of pain relief.

If the placebo has stimulated release of mu-specific ligands, there should be a corresponding drop in specific binding of [^{11}C]carfentanyl because of the competitive effect of the endogenous ligands. Brain areas particularly affected should include those areas enriched in mu receptors (for example, the periaqueductal gray matter and the thalamus). The data could be analyzed according to conventional PET qualitative bind-

ing study methods, such as those described by Raichle and co-workers.[26]

References

1.) Akil, H., Watson, S., Young, E., Lewis, M., Khachaturian, H., and Walker, M. (1984) *Ann. Rev. Neurosci.* **7**, 233-255

2.) Mayer, D., Wolfe, T., Akil, H., and Carder, B. (1971) *Science* **174**, 1351-54

3.) Akil, H., Mayer, D., and Liebeskind, J. (1972) *CR Acad. Aci Paris* **274**, 3603-5

4.) Mayer, D., and Hayes, R. (1975) *Science* **188**, 941-43

5.) Akil, H., and Hughes, J., Richardson, D., and Barchas, J. (1978) *Science* **201**, 463-465

6.) Akil *et al* (1984)

7.) *ibid*

8.) *ibid*

9.) Seeger, T., Sforzo, G., Pert., C., and Pert, A. (1984) *Brain Res* **305**, 303-311

10.) Akil *et al* (1984)

11.) Seeger *et al* (1984)

12.) Akil *et al* (1984)

13.) Martin, W., Eades, C., Thompson, J., Huppler, R., and Gilbert, P. (1976) *J. Pharmacol. Exp. Ther.* **197**, 517-532

14.) Frost, J.J., Dannals, R., Duelfer, T., Burns, H., Ravert, H., Langstrom, B., Balasubramanian, V., and Wagner, H. (1984) *Ann. Neurol.* **15**, S85-S92

15.) Pfeiffer, A., Pasi, A., Mehraein, P., and Herz, A. (1982) *Brain Res.* **248**, 87-96

16.) Goldstein, A. (1987) *TIPS* **8**, 456-59

17.) Frost, J.J. (1986) *TIPS* **7**, 490-96

18.) *ibid*

19.) *ibid*

20.) Frost *et al* (1984)

21.) Frost, J.J., Behal, R., Mayberg, H., Dannals, R., Ravert, H., Wilson, A., Links, J., and Wagner, H. (1986) *J. Nucl. Med.* **27**, P235

22.) Frank, J.D. (1973) *Persuasion and Healing.* The Johns Hopkins University Press

23.) Cousins, N. (1981) *Anatomy of an Illness as Perceived by the Patient.* Bantam Books

24.) *ibid*

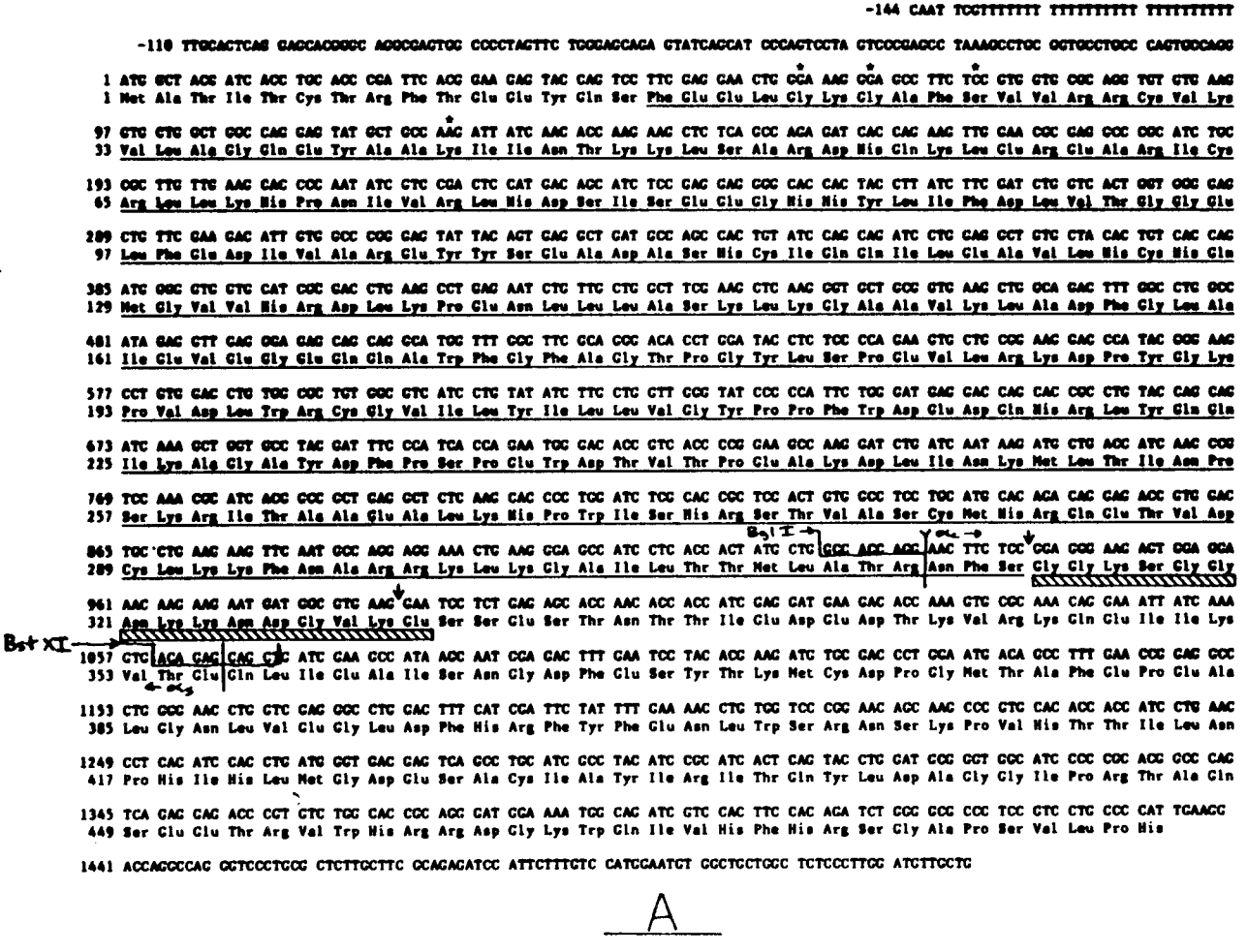
25.) *ibid*

26.) Mintun, M., Raichle, M., Kilbourn, M., Wooten, G., and Welch, M. (1983) *Ann. Neurol.* **15**, 217-27

Proposed Investigations of the Binding Properties of a Possible PSD-binding Domain in the Alpha Subunit of the Type II CaM Kinase

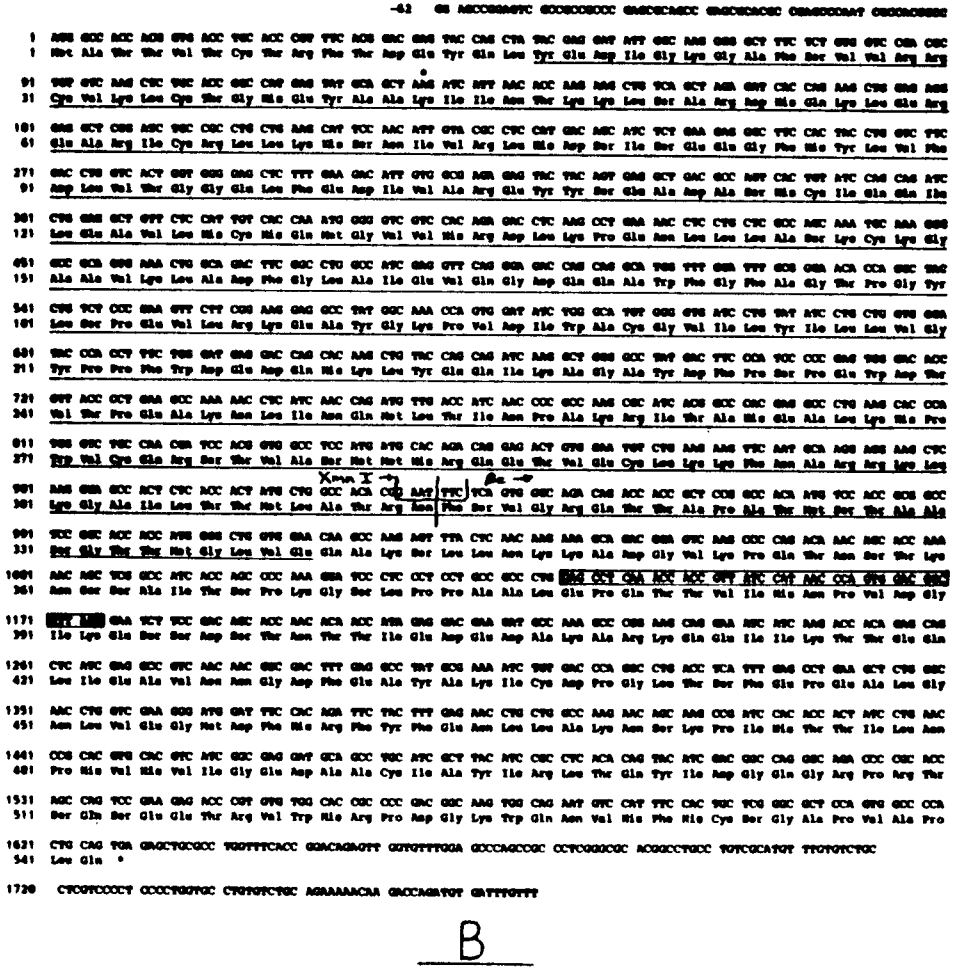
The Type II Ca^{2+}/calmodulin-dependent protein kinase (Type II CaM kinase) is an enzyme which is expressed almost exclusively in the brain, is particularly concentrated in PSD's, and has a rather broad substrate specificity.[1] It is capable of a calcium-dependent autophosphorylation which switches the kinase into a state which then allows calcium independent phosphorylation of itself and exogenous substrates; this phenomenon may endow the kinase with the potential to be regulated by changes in neuronal calcium concentrations, and consequently may also allow it to participate in relatively long-term changes in response to neuronal firing.[2] The kinase exists as a large holoenzyme which is comprised of from ten to twelve subunits of two related types, which are referred to as the "alpha" and "beta" subunits, and which are present in varying proportions in different isozymes of the holoenzyme.[3] One isozyme predominates in the forebrain, and consists of nine alpha subunits (of 50 KD each) and three beta subunits (of 60 KD each), while another isozyme predominates in the cerebellum, and apparently contains eight beta and two alpha subunits.[4] The two types of subunits are extensively homologous, can both bind calmodulin and ATP, and are both capable of autophosphorylation.[5] The most highly conserved regions include the "kinase domain" which is similar to regions present in all kinase proteins, contains the putative ATP binding site, and is located within the amino-terminal half of both subunits (see figs. A and B); also the calmodulin-binding domain, which is identical in the two subunits, and is located somewhat downstream of the kinase domain.[6] The carboxy termini are a bit less highly conserved (76% compared to 91% for the amino half), and there is no similarity between these domains and those of other known protein kinases, and their regulatory roles are uncertain.[7] The major difference between the subunits is the deletion of two segments from alpha which are present in beta, and which are located near the junction of the amino and carboxy halves.[8] The known functions of the subunits are generally very similar, with the only notable exc-

α subunit


-144 CAAT TCGTTTTTTT TTTTTTTTTT TTTTTTTTTT
-110 TTGCACTCAG GAGCACGGGC AGGCGAGTGG CCCCTAGTTC TGGGAGCAGA GTATCAGCAT CCCAGTCCTA GTCCCGAGCC TAAAGCCTGC GGTGCCTGCC CAGTGGCAGG
1 ATG GCT ACG ATC ACC TGC ACC CGA TTC ACG GAA GAG TAC CAG TCC TTC GAG GAA CTG GCA AAG GCA GCC TTC TCC GTG GTG CGC AGG TGT GTG AAG
1 Met Ala Thr Ile Thr Cys Thr Arg Phe Thr Glu Glu Tyr Gln Ser Phe Glu Glu Leu Gly Lys Gly Ala Phe Ser Val Val Arg Arg Cys Val Lys
97 GTG CTG GCT GCC CAG GAG TAT GCT GCC AAC ATT ATC AAC ACC AAG AAG CTC TCA GCC ACA GAT CAC CAG AAG TTG GAA CGC GAG GCC CGC ATC TCC
33 Val Leu Ala Gly Gln Glu Tyr Ala Ala Lys Ile Ile Asn Thr Lys Lys Leu Ser Ala Arg Asp His Gln Lys Leu Glu Arg Glu Ala Arg Ile Cys
193 CGC TTG TTG AAG CAC CCC AAT ATC GTC CGA CTC CAT GAC AGC ATC TCC GAG GAG GGC CAC CAC TAC CTT ATC TTC GAT CTG GTC ACT GGT GGC GAG
65 Arg Leu Leu Lys His Pro Asn Ile Val Arg Leu His Asp Ser Ile Ser Glu Glu Gly His His Tyr Leu Ile Phe Asp Leu Val Thr Gly Gly Glu
289 CTG TTC GAA GAC ATT GTG GCC CGC GAG TAT TAC AGT GAG GCT GAT GCC AGC CAC TGT ATC CAG CAG ATC CTG GAG GCT GTC CTA CAC TGT CAC CAG
97 Leu Phe Glu Asp Ile Val Ala Arg Glu Tyr Tyr Ser Glu Ala Asp Ala Ser His Cys Ile Gln Gln Ile Leu Glu Ala Val Leu His Cys His Gln
385 ATC GGC GTC GTG CAT CGC GAC CTG AAG CCT GAG AAT CTG TTG CTG GCT TCG AAG CTC AAG GGT GCT GCC GTG AAG CTC GCA GAC TTT GGC CTG GCC
129 Met Gly Val Val His Arg Asp Leu Lys Pro Glu Asn Leu Leu Leu Ala Ser Lys Leu Lys Gly Ala Ala Val Lys Leu Ala Asp Phe Gly Leu Ala
481 ATA GAG GTT GAG GGA GAG CAG CAG GCA TGG TTT GGC TTC GCA GGC ACA CCT GGA TAC CTC TCC CCA GAA GTG CTG CGC AAG GAC CCA TAC GGG AAG
161 Ile Glu Val Glu Gly Glu Gln Gln Ala Trp Phe Gly Phe Ala Gly Thr Pro Gly Tyr Leu Ser Pro Glu Val Leu Arg Lys Asp Pro Tyr Gly Lys
577 CCT GTG GAC CTG TGG GCC TGT GGC GTC ATC CTG TAT ATC TTC CTC GTT GGG TAT CCC CCA TTC TGG GAT GAG GAC CAG CAC CGC CTG TAC CAG CAG
193 Pro Val Asp Leu Trp Arg Cys Gly Val Ile Leu Tyr Ile Leu Leu Val Gly Tyr Pro Pro Phe Trp Asp Glu Asp Gln His Arg Leu Tyr Gln Gln
673 ATC AAA GCT GGT GCC TAC GAT TTC CCA TCA CCA GAA TGG GAC ACC GTC ACC CCG GAA GCC AAG GAT CTG ATC AAT AAG ATG CTG ACC ATC AAC CCG
225 Ile Lys Ala Gly Ala Tyr Asp Phe Pro Ser Pro Glu Trp Asp Thr Val Thr Pro Glu Ala Lys Asp Leu Ile Asn Lys Met Leu Thr Ile Asn Pro
769 TCC AAA CGC ATC ACG GCC GCT GAG GCT CTC AAG CAC CCC TGG ATC TCG CAC CGC TCC ACT GTG CCC TCC TGC ATG CAC AGA CAG GAG ACC GTG GAC
257 Ser Lys Arg Ile Thr Ala Ala Glu Ala Leu Lys His Pro Trp Ile Ser His Arg Ser Thr Val Ala Ser Cys Met His Arg Gln Glu Thr Val Asp
BglI
865 TGC CTG AAG AAG TTC AAT GCC AGC AGG AAA CTG AAG GCA GCC ATC CTC ACC ACT ATG CTG GCC AGC AGC AAC TTC TCC GGA CGG AAC ACT GGA GGA
289 Cys Leu Lys Lys Phe Asn Ala Arg Arg Lys Leu Lys Gly Ala Ile Leu Thr Thr Met Leu Ala Thr Arg Asn Phe Ser Gly Gly Lys Ser Gly Gly
961 AAC AAG AAG AAT GAT GGC GTG AAC GAA TCC TCT GAG AGC ACC AAC ACC ACC ATC GAC CAT GAA GAC ACC AAA GTG CGC AAA CAG GAA ATT ATC AAA
321 Asn Lys Lys Asn Asp Gly Val Lys Glu Ser Ser Glu Ser Thr Asn Thr Thr Ile Glu Asp Glu Asp Thr Lys Val Arg Lys Gln Glu Ile Ile Lys
BstXI
1057 GTC ACA GAC CAG CTC ATC GAA CCC ATA AGC AAT CCA GAC TTT GAA TCC TAC AGC AAG ATC TGC GAC CCT GCA ATG ACA GCC TTT GAA CGG GAG GCC
353 Val Thr Glu Gln Leu Ile Glu Ala Ile Ser Asn Gly Asp Phe Glu Ser Tyr Thr Lys Met Cys Asp Pro Gly Met Thr Ala Phe Glu Pro Glu Ala
1153 CTC GGC AAC CTC GTC GAG GGC CTC GAC TTT CAT CCA TTC TAT TTT GAA AAC CTG TGG TCC CGC AAC AGC AAG CCC GTC CAC ACC ACC ATC CTG AAC
385 Leu Gly Asn Leu Val Glu Gly Leu Asp Phe His Arg Phe Tyr Phe Glu Asn Leu Trp Ser Arg Asn Ser Lys Pro Val His Thr Thr Ile Leu Asn
1249 CCT CAC ATC CAC CTG ATG GGT GAC GAG TCA GCC TGC ATC GCC TAC ATC CGC ATC ACT CAG TAC CTG GAT GCG GGT GGC ATC CCC CGC ACG GCC CAG
417 Pro His Ile His Leu Met Gly Asp Glu Ser Ala Cys Ile Ala Tyr Ile Arg Ile Thr Gln Tyr Leu Asp Ala Gly Gly Ile Pro Arg Thr Ala Gln
1345 TCA GAG GAC ACC CCT GTC TGG CAC CGC AGG CAT CGA AAA TGG CAG ATC GTC CAC TTC CAC AGA TCT CGG GCG CCC TCC CTC CTG CCC CAT TGAAGG
449 Ser Glu Glu Thr Arg Val Trp His Arg Arg Asp Gly Lys Trp Gln Ile Val His Phe His Arg Ser Gly Ala Pro Ser Val Leu Pro His
1441 ACCAGGCCAG GGTCCCTGCG CTCTTGCTTC GCAGAGATCC ATTCTTTGTC CATGCAATGT GGCTGCTGCC TCTCCCTTGC ATCTTGCTG

A

β subunit



B

eption being that the affinity of the different kinase isozymes for PSD's may be related to the proportion of alpha to beta subunits within an isozyme; it appears that a higher ratio of alpha to beta leads to a greater association of the enzyme with PSD's.[9] If this functional difference is related to the only major structural differences in the subunits, then the unique domain in alpha (made possible by the deletions from beta) may be responsible for binding the alpha subunit (and hence the holoenzyme) to the PSD. This possibility seems more likely in consideration of the fact that regions which are brought together in alpha are highly charged and hydrophilic, while the deleted interstitial segments are mostly neutral and are predicted to have a relatively random structure.[10]

The hypothesis which will be addressed in this proposal is that the domain in the alpha subunit which is brought together by deletions relative to the beta subunit may be responsible for the binding of the kinase holoenzyme to postsynaptic densities.

I propose that this hypothesis can be tested by using peptides which are identical to portions of the carboxy-terminal halves of the alpha and beta subunits. One of these peptides would be from the alpha subunit, and would include largely the potential PSD-binding domain -- the section beginning at the end of the calmodulin-binding domain, and ending about 30 amino acid residues downstream of the point of the second "deletion" from the beta subunit. I will refer to this peptide as "alpha$_S$", for "short alpha segment." The other two peptides would be basically the carboxy-terminal halves of the alpha and beta subinits, and would be used to compare the PSD-binding properties of the subunits while excluding regions of known functionality. I will refer to these as alpha$_C$ and beta$_C$ for "alpha- and beta-C-termini." In order to produce amounts of the peptides large enough for experimentation, a system which allows the expression of eukaryotic proteins in *E. coli* will be used. This method creates "fusion proteins" which are linkages of proteins of bacterial and eukaryotic origin, and has

been used to successfully express several eukaryotic proteins in *E. coli.*[11] Separation of the protein of interest from the bacterial protein by proteolytic digestion can be accomodated by the engineering of a protease recognition site or chemical cleavage site within the cloning vector.[12]

Preliminary PSD binding studies of the peptides would be done by incubating them with nitrocellulose blots of PSD fractions. PSD proteins which bound the peptides most strongly would then be isolated and would be sequenced in a manner very similar to the methods discussed for the proteins in the research report which accompanies this proposal. After the sequence of a particular protein is obtained, further binding studies between the protein and the CaM kinase peptides could be performed in order to determine the specific domains in the PSD protein responsible for the binding. This could be done following production of large amounts of specific portions of the PSD protein via an *E. coli* fusion protein expression system.

Experimental Setting

Until fairly recently, it was generally very difficult to obtain high-level expression of eukaryotic proteins in bacterial systems, possibly because the proteins are recognized as foreign and are degraded[13] or because of general differences in coding regions between bacterial and eukarytic DNA. Recent advances have lessened these difficulties considerably, however. The general method used is designed to create "fusion proteins" made from combinations of bacterial and eukarytic DNA. Also included in the cloning vectors are sequences coding for recognition sites of proteases or chemical cleaving reagents, which are placed between the two types of protein so that separation of the pieces can be easily accomplished. The eukaryotic DNA is spliced into the vector so as to be translated after the bacterial DNA for the fusion protein, and must be situated so that it will be read in-phase, with the accomodations for the inter-

vening code for the protease recognition site. Several different proteases and chemical cleaving reagents have been used for the splitting the fusion proteins[14]; the general requirement is that the eukaryotic protein of interest may not contain an internal recognition site for the proposed cleaving agent. For the proposed expression of the kinase peptides, cyanoogen bromide (which cleaves between Met--Xaa) is suitable; this cleaving agent was, for example, used in the production of human insulin via the plasmid vector pBR322.[15] In that particular system, the gene for insulin was ligated to the *E. coli* gene for beta-galactosidase, along with the "AUG" coding for the Met site with which CNBr reacts. Genetic manipulations were performed in order to splice the genes in the Eco Rl-Bam Hl region of pBR322. This vector and strategy would also be appropriate for the cloning of fusion proteins between beta-galactosidase and the CaM kinase peptide fragments. The DNA encoding an entire kinase subunit could be cleaved at approipriate sites by restriction endonucleasees; the correct DNA fragment could be joined to beta-gal by blunt-end ligation techniques, and to the plasmid after adding a Bam Hl fragment onto the fragment. Fortunately, commercially-available restriction endonucleases exist which can cleave the kinase alpha and beta subunits within the desired regions. Creation of the $alpha_C$ and $beta_C$ DNA fragments would require only one cut, preferably within the calmodulin binding domain (or, at least, as close to the amino-terminal side of the carboxy half as possible). For the alpha subunit, the calmodulin binding domain is from bases 887-942; the endonuclease"Bgl I" cuts at 145 and 934 (see fig. A). Fot the beta subunit, the calmodulin binding domain is from bases 890-945; the endonuclease "Xmn I" cuts at 616 and 939 (see fig. B). Thus the $alpha_C$and $beta_C$ peptides would contain essentially only the carboxy termini, with only the addition of a small portion of the calmodulin binding domain. Creation of the $alpha_S$ DNA is easily accomplished by cutting the $alpha_C$ DNA with"Bst XI" which cleaves at

1065 (leaving the $alpha_S$ fragment of 131 base pairs and a disposable 3' terminus of 607 base pairs). The $alpha_S$ DNA would code not only the region which is altered from beta, but also an additional thirty residues to the carboxyl side of it as well. This would allow for possibly folding which may depend on nearby domains, as well as allowing for the possibility that part of the PSD binding domain was in the additional segment as well as the "alpha-deletion" segment.

Theoretically, it seems that the kinase peptides could be simply obtained by using a fusion protein expression system utilizing an appropriate plasmid vector and bacterial strain, and then cleaving the peptides from beta-galactosidase with CNBr. However, a problem that usually occurs with these systems is that the highly-expressed fusion protein is produced in the form of an insoluble, inactive"inclusion complex."[16] Fortunately, the inclusion bodies are comprised largely of recombinant protein, and purification methods have been developed which can remove contaminants by detergent solubilization, and then dissolve the inclusion bodies in strong denaturants, after which the protein is allowed to refold in a correct manner by replacement of the strong denaturant with a weaker one.[17] First, the bacterial cells must be lysed so that the inclusion bodies can be isolated. Contaminants can be largely removed by dissolving them in a detergent such as Triton. The inclusion bodies are then solubilized in a strong denaturant, such as 8 molar guanidinium chloride, strong alkiline solutions, or 6-8 molar urea. During the refolding stage, the concentration of the protein and denaturing agents, as well as the pH, must be controlled carefully to optimize protein yield and to achieve proper refolding. At this point, the fusion protein can then be subjected to the protease digestion or to chemical cleavage to separate the component proteins. Alternatively, the digestion/cleavage step may be performed after denaturation and before refolding.

Now that the kinase peptide fragments have been produced in large yield, they

can be used in binding studies. These binding studies would be qualitative experiments designed to identify PSD proteins which may be responsible for allowing the CaM kinase holoenzyme to bind to PSD's. The PSD proteins would be separated on a gel, and then transferred to nitrocellulose. These transfer blots would then be incubated individually with each of the kinase peptides, which would be radiolabelled with ^{125}I via a Bolton-Hunter reagent. In binding studies similar to these, Sahyoun *et al* have preliminary findings indicating that a group of proteins of M_r's of about 140 KD can bind to intact alpha subunits of the Type II CaM kinase.[18]

After a protein is identified which binds to the $alpha_S$ or $alpha_C$ peptides, it can then be sequenced using the same methods which were described in the research report which accompanies this proposal. The protein would be transferred to nitrocellulose and subjected to a trypsin digest, after which the tryptic fragments could be sequenced by HPLC and several fragments sequenced. The known sequences of the fragments could then be used to design synthetic oligonucleotides which would then be used to select appropriate DNA from a rat brain cDNA library. The cDNA would be sequenced and the protein's amino acid sequence inferred.

At this stage the *E. coli* fusion protein expression system could again be used, this time to generate large amounts of peptide fragments included in the sequence of the protein chosen for study. These fragments could then be used in further binding studies, this time with the goal of determining the specific domain of the PSD protein which was responsible for the protein's binding to the kinase peptides in the earlier binding studies. In this way, through a systematic study of various domains of the PSD protein being studied, it may be possible to determine the specific protein interactions required for binding of the CaM kinase to PSD's.

Figure Legends

Fig. A: Peptide and nucleotide sequence of the alpha subunit of Type II CaM kinase; underlined nucleotides indicate recognition sequences of Bgl I and Bst XI; lines between bases and peptides indicate sites of the endonucleases. The hatched bar beneath the peptide sequence indicates the hypothetical PSD-binding domain.

Fig. B: Peptide and nucleotide sequence of the beta subunit of Type II CaM kinase; with recognition and cleavage sites of Xmn I marked as in Fig. A. The long underlined region is the kinase activity domain.

References

1.) Bennett, M.K., Erondu, N.E., and Kennedy, M.B. (1983) *J. Biol. Chem.* **258**, 12735-44

2.) Bennett, M.K. and Kennedy, M.B. (1986) *Proc. Natl. Acad. Sci. USA* **84**, 1794-98

3.) Bennett *et al* (1983)

4.) Miller, S.G. and Kennedy, M.B. (1985) *J. Biol. Chem.* **260**, 9039-46

5.) Bulleit, R.F., Bennett, M.K., Molloy, S.S., Hurley, J.B., and Kennedy, M.B. (1988) *Neuron* **1**

6.) *ibid*

7.) *ibid*

8.) *ibid*

9.) Miller and Kennedy (1985)

10.) Bulleit *et al* (1988)

11.) Marston, F.A.O. (1986) *Biochem. J.* **240**, 1-12

12.) *ibid*

13.) *ibid*

14.) *ibid*

15.) Goeddel, D.V., Kleid, D.G., Bolivar, F., Heyneker, H.L., Yansura, D.G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K., and Riggs, A.D. (1979) *Procl. Natl. Acad. Sci. USA* **76**, 106-110

16.) Marston (1986)

17.) *ibid*

18.) Sahyoun, N., Levine, H., McDonald, B., and Cuatrecasas, P. (1986) *J. Biol. Chem.* **261**, 12339-44