1994

Nerd: A Neuron-Specific Gene in Drosophila Melanogaster Related to Vertebrate GAP-43

Victor Neel

nerd: A neuron-specific gene in *Drosophila melanogaster* related to vertebrate GAP-43

A thesis submitted to the faculty of Rockefeller University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

by
Victor Allen Neel

March 1994
New York

For Lila, Seth and Dylan

Acknowledgements

I am blessed to have been surrounded by caring individuals who have bolstered my flagging ego and given me thoughtful philosophies to replace failed experiments and moments of desperation. I would have been far too easy to allow my spirits to rise and fall with the vagaries of the graduate and medical school "experience." Instead these kind folk have anchored me to a much larger existence.

I wish to thank the members of the Young lab for their selfless gifts of time and scientific know-how. Toby Lieber has been simply invaluable to my work. Simon Kidd never let me forget the place of my work in a larger scheme. Liz Alcamo, Terry Evering and Hank Radiewicz did the majority of the mutant collection. Cedric Wesley restored my faith in PCR and gave freely of his expertise in evolution. The *per* crew, Amita Sehgal, Jeff Price, Mike Myers, Lei Rong, Mary Baylies and Lino Saez, were always encouraging and helpful. My fellow grad students, Adrian Rothenfluh,Leslie Vosshall and Amy Kiger provided the right mix of humor and encouragement to make the bleakest days tolerable.

Several scientists have been role models for me and I am duly thankful: Alan Aderem for his electric enthusiasm, Tim Tully for his calm and unwavering vision, and especially Michael Young, my advisor, for his protean knowledge of genetics and his faith in me. Mike gave me the precious gift of scientfic freedom, from which self-confidence and integrity have sprung. If I succeed as a scientist it will be without doubt be in part from his efforts. Of course if I fail I hold him completely responsible.

My brothers, Ben, Brad and David, and my sister-in-law Phyllis have helped to offset the hard times.

Alan Swartz has been a sharp critic and my most accepting friend, though we don't hug.

Dehan, Oshana and Chuti have unfailingly provided our home with love and have dealt deftly with my moody, irascible ways.

Ammi and Thathi have given us so much I cannot begin to catalog their generosity. The most invaluable gift has been a trust with their dearest treasure.

I would not be able to express my love for Lila, Seth and Dylan without sounding trite. It must suffice to say that without the three of them I could not be.

Table of Contents

List of Figures

Abstract

The neuronal protein GAP-43 has been implicated in a number of key processes in neural development and synaptic behavior. There have been no reports identifying an invertebrate molecule with homology to GAP-43. In this study we have begun to explore the biochemistry and genetics of the *Drosophila* gene *nerd*, which was isolated by vitrue of its DNA homology to mammalian GAP-43.

Two proteins arise from the *nerd* locus and share a conserved motif, the GAP43 module. This twenty amino acid domain unites a small family of brain-specific mammalian proteins that are believed to function similarly. Based on protein sequence, biochemical activities and nervous system-specific tissue distribution we argue that *nerd* represents a functional homolog of vertebrate GAP-43.

The possibility of a mutational analysis of *nerd* makes the study of this gene in *Drosophila* a valuable extention of previous studies in vertebrate systems. An initial characterization of mutants that map within or close to the *nerd* region on the second chromosome is detailed.

CHAPTER I: Overview and Background

Overview

A startling revelation in neurobiology research has taken form over the last three decades. The intricate structure of the adult brain, with its hundreds of trillions of connections, is constantly evolving from moment to moment. This web-like lattice is plastic, distilling the essence of the information it receives from the senses into subtle variations in its structure. These changes take the form of alterations in the shape and extent of dendritic arborizations and variations in the strength of coupling between neurons. The remodeling of neuronal contacts is believed to underlie the processes of learning and memory storage.

Evolution has been frugal, and memory formation makes use of many of the same mechanisms that are called into play during neuronal development. In addition, the primary signal transduction pathways have been highly conserved at the molecular and cellular level from species to species. Studies in model systems have highlighted the similarities between the synaptic physiology of the marine invertebrate *Aplysia* and hippocampal preparations from the rat. The fruitfly has also been useful in examining the molecular details of learning and memory. Mutations interrupting the aquisition and retrieval of learned behaviors have confirmed the importance of key regulators identified in other systems.

Through the efforts of many groups the vertebrate protein GAP-43 has taken a prominent place in molecular neurobiology. It is associated with a range of synaptic behaviors, including neurite outgrowth, neurotransmitter release and long-term potentiation (LTP). The specific function of GAP-43 in these processes remains a mystery. With the goal of establishing a genetic system to study the function of GAP-43, the specific aim of this work was to identify a gene product

functionally related to GAP-43 in *Drosophila melanogaster* and initiate a genetic screen to uncover lethal mutations in the gene.

Background

This section details the history of GAP-43 in neurobiology. The same protein has been studied by several groups investigating different processes, and the nomenclature resulting from the overlap tends to be confusing. The following names all refer to the same protein : GAP-43, GAP-48,pp46, B-50, F1, and neuromodulin (P-57). A brief description of each group's research and their co-discovery of GAP-43 is useful since it highlights the different facets of GAP-43 biology.

A 43kd Growth-Associated Protein

Lower vertebrates, and to a lesser extent mammals, have a spectacular capacity to regenerate functional connections following nerve injury and subsequent axon retraction. This principle was demonstrated by Sperry in his classic experiments on amphibian optic nerve regeneration. Within weeks of the experimental lesioning, the optic nerve can retrace its path and establish a precise retinotopic map in the tectum, eventually restoring complete function to the animal. In an attempt to identify the proteins that mediate this phenomenon, Skene and Willard (Skene, 1981) (Skene and Willard, 1981a) identified proteins in the toad optic nerve with dramatically elevated expression during the regenerative phase. The expression of one of these proteins, a membrane-associated protein with a molecular weight of 43kD on SDS-PAGE gels, was rapidly induced and remained at high levels until after the period of functional recovery, when it declined to baseline levels. Since the detection of this protein was coincident with the period of axon elongation and pathfinding, they

proposed that Growth-Associated Protein-43kD, or GAP-43, was involved in these processes. They also found a protein with a similar molecular weight and acidic pI in the adult hypoglossal nerve and neonatal optic nerve, both of which have the capacity to regenerate. In contrast, in adult rabbit retinal cells, which have lost their regenerative potential, GAP-43 expression was not induced following injury. Benowitz *et al.* (Benowitz et al., 1981) reported the appearance of a similar, rapidly transported protein, GAP-48, in the regenerating goldfish optic nerve.

It is widely held that regeneration is a recapitulation of ontogeny. Since the original characterizations of GAP-43, numerous studies have detailed its expression during development as well as in experimentally-induced injury. In the developing rat spinal cord, GAP-43 immunoreactivity is first seen on embryonic day 11(E11) staining cell bodies of dorsal root ganglia and motorneurons as well as axons in the dorsal and ventral horns (Fitzgerald et al., 1991). The staining pattern shifts over time away from cell bodies to become predominantly axonal. A reduction in expression is tightly correlated with the innervation of peripheral targets, when synaptogenesis is taking place. In the cerebral cortex, axons in the subplate region express moderate levels of GAP-43 at E17, but a large increase is seen during the first postnatal week (Erzurumlu et al., 1990), particularly in the barrel fields of the somatosensory cortex. These levels decline sharply shortly thereafter. The rise and fall in expression overlaps a developmental "critical period," a phase where peripheral inputs are generally competing for cortical representation (Belford and Killackey, 1980) (Rakic, 1981), *i.e.* establishing functional synapses. A similar rise and fall of GAP-43 expression takes place in the cat striate cortex when ocular dominance columns arise (Benowitz et al., 1989).

The regenerative potential of a particular neuron following axotomy is linked to its ability to express GAP-43. This correlation has been demonstrated repeatedly (Knyihar-Csillik et al., 1992). A vivid example is seen in Doster *et al.* (Doster et al., 1991). Under normal circumstances the adult mammalian optic nerve is unable to regenerate following transection. The damaged axons retract and the retinal ganglion cells die shortly thereafter. GAP-43 levels remain very low following this procedure. However, if a peripheral nerve graft is interposed adjacent to the lesion, a partial regeneration of the axon takes place and the severed axons "grow" into the graft. Coincident with this restored outgrowth, GAP-43 immunoreactivity explodes (see Figure I.1). Successful regeneration and GAP-43 expression go hand in hand.

It appears that all neurons express GAP-43 during an early period in their development, for wherever investigators care to look for it, it is detected. No report in the literature describes a collection of neurons that, when followed through development, do not express GAP-43. Once synaptogenesis has taken place, expression is markedly reduced, except in discrete regions in the adult (Neve et al., 1988).

Synaptic plasticity, LTP and phosphorylated GAP-43

One hallmark of the nervous systems of advanced organisms is their plasticity, or competence to alter the strength of connections in an activity-dependent fashion. Originally described by Bliss and Lomo (Bliss and Lomo, 1973), long-term potentiation (LTP) is defined as a sustained increase in transmission at the synapse following previous activity. As such, it has been proposed to underlie learning and memory formation. The most common substrate for experimental manipulation of LTP is the hippocampus. The highly stereotypical anatomy of the region presents an appealing experimental model.

Figure I.1: GAP-43 expression is induced following injury

This figure, taken from Doster *et al.* (Doster et al., 1991), is a superb example of the explosive induction of GAP-43 following damage to the rabbit optic nerve. While most neurons of the mammalian CNS are unable to initiate neurite outgrowth following injury, if the lesion is placed very close to the orbit, the optic nerve can partially regenerate. The ability of the nerve to initiate this process is strictly correlated with GAP-43 expression. [The presence of GAP-43 (the white signal in the left panel) is detected only with the proximal lesion.]

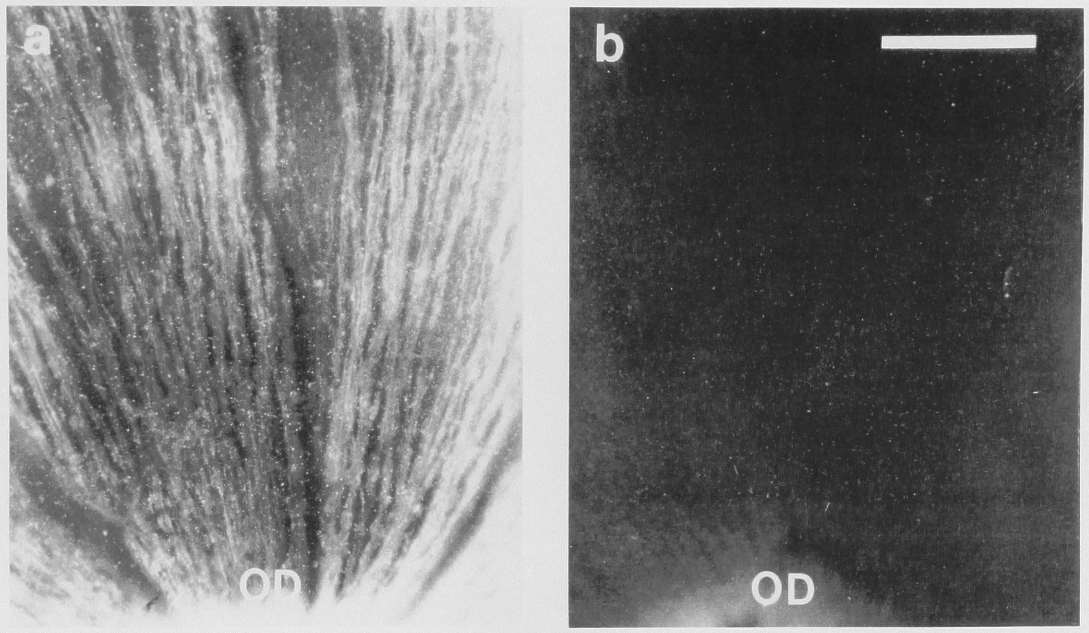


Figure 4. GAP-43 Immunofluorescence in Axotomized and Control Retinas

Anti-GAP-43 (5.6 μg/ml) binding to whole mounts of adult rat retina 12 days after axotomy within 3 mm of the eye (a) compared with a control retina (unoperated side; b). OD, optic disk. Bar, 200 μm.

Axons of pyramidal cells in the CA3 field determine the Schaffer collateral pathway and synapse on their target neurons in the CA1 region. Stimulation of the CA3 field leads to the production of LTP in CA1 postsynaptic cells (Malenka and Nicoll, 1993).

While the mechanisms that generate LTP are still hotly debated and beyond the scope of this study, several points are generally agreed upon. Both pre- and postsynaptic elements are important for the full expression of LTP (Ben-Ari et al., 1992). The presynaptic component may require production of a retrograde messenger to communicate postsynaptic activation (O'Dell et al., 1991; Fazeli, 1992, but see Cormier et al., 1993). In addition, the persistent activation of protein kinases is important in some forms of LTP (Malinow et al., 1988).

Routtenberg and colleagues initially set out to isolate brain phosphoproteins (Ehrlich and Routtenberg, 1974) with the aim of correlating alterations in phosphorylation with behavior (learning) and cellular changes (LTP). Using an intact hippocampal formation and a *post hoc in vitro* phosphorylation assay, the appearance of an acidic, 47kD phosphoprotein called F1, was consistently associated with LTP (Routtenberg et al., 1985).

The kinase responsible for interacting with F1 was quickly determined to be protein kinase C, a then newly discovered kinase that had a calcium and phospholipid cofactor requirement (Nishizuka, 1986). Gispen and his followers were also interested in the PKC-dependent phosphorylation (Aloyo et al., 1983) of a brain-specific 50kD protein whose phosphorylation was *inhibited* by the behaviorally-active adrenocorticotrophic hormone (ACTH) peptide (Zwiers et al., 1978). The protein, called B-50, was concentrated in synaptic plasma membrane fractions and was eventally shown to reside presynaptically (Gispen et al., 1985).

Was F1/B-50 a synapse-specific protein, or was it present during development? The presynaptic membrane is a derivative of the growth cone. In

order to evaluate the developmental history of F1/B-50, Routtenberg collaborated with Pfenninger (Katz et al., 1985), who had been studying pp46, a calcium/phospholipid dependent phosphoprotein enriched in fetal growth cone preparations. Partial proteolytic mapping suggested the identity of pp46 with F1. The simultaneous molecular cloning of GAP-43 in two laboratories (Basi et al., 1987; Karns et al., 1987), and F1 (Rosenthal et al., 1987) relieved any suspicions that all the proteins were not identical.

The potential implications of the finding that a "synaptic plasticity" protein and a "growth-associated" protein were one and the same was appealing. The notion that synaptic plasticity might ultimately be a form of presynaptic growth had been suggested earlier (Lee et al., 1980; Carlin and Siekevitz, 1983).

GAP-43 as a calmodulin-binding protein

In 1983, Storm's group published a report concerning the purification of a novel protein with unusual CaM-binding properties (Andreasen et al., 1983). The calcium signal in cells is frequently transmitted through the activation of enzymes and proteins that associate with Ca^{+2}/CaM. In the absence of Ca^{+2}, the affinity of the molecules for CaM, and hence their activity, is low. A very abundant membrane associated 57kD (known as P-57) protein from bovine cerebral cortex displayed the opposite characteristics, namely, CaM association in the absence of calcium (Ca^{+2}) and dissociation in the presence of Ca^{+2}. Based on this observation alone, a function for P-57 was immediately suggested. The protein could serve as a CaM "store", releasing it under conditions of high intracellular calcium. Free CaM would then proceed to activate CaM-dependent enzymes. Intriguingly, PKC phosphorylation of P-57 abolished CaM binding. This was shown to result from the proximity of the PKC-site and the CaM-binding site (Alexander et al., 1987; Alexander et al., 1988). The cDNA sequence

from P-57 appeared in print the same year as GAP-43 (Cimler et al., 1987), confirming their identity.

GAP-43 has been multiply discovered as a prominent CaM-binding, neuronal phosphoprotein involved with neurite outgrowth and synaptic plasticity. In the following sections I describe work aimed at determining a function for this protein.

Growth cone motility and GAP-43

Because of it temporal expression pattern, the notion has arisen that GAP-43 *mediates* neurite outgrowth. A recent experimental finding challenges this idea. PC12 cells are a tumor cell line from a rat pheochromocytoma (adrenal) culture and have been used extensively as a model for neuronal differentiation (Greene and Tischler, 1976). The cells express GAP-43 at high levels when they are induced to differentiate in the presence of nerve growth factor (NGF) (Costello et al., 1987). In a PC12 cell line selected for having undetectable levels of GAP-43, neurite outgrowth is intact following NGF challenge (Baetge and Hammang, 1991). This suggests that outgrowth *per se* does not require GAP-43, but does not eliminate the possibility that GAP-43 might have an important modulatory role in the outgrowth process.

In a recent paper, Aigner and Caroni (Aigner and Caroni, 1993) explanted embryonic dorsal root ganglia and blocked production of GAP-43 protein with antisense RNA. They found that the development of neurites could proceed, but that this capacity was substrate-sensitive. On laminin, thin calibered neurites with small growth cones appeared in GAP-43(-) explants (reminiscent of Baetge and Hammang (Baetge and Hammang, 1991)) whereas GAP-43(+) neurites and growth cones were much larger. Moreover, on a poly-L-ornithine substrate, GAP-43(-) neurons failed to produce neurites altogether. Previously, Widmer and Caroni (Widmer and Caroni, 1993) had transfected nonneuronal spreading

L6 cells (a myogenic cell line) with either a GAP-43 mutant cDNA that prevented the phosphorylation by PKC (GAP-43(Ala)) or a GAP-43 cDNA that mimicked constitutive phosphorylation (GAP-43(Asp)). In GAP-43(Ala)-transfected cells, there was poor spreading and few filopodial structures, whereas GAP-43(Asp)-transfected cell lines spread well and had large growth cone-like structures. One conclusion to be drawn from these experiments is that the ability of growth cones to respond to local stimuli is conditionally dependent on the presence of GAP-43. In a "rich" extracellular environment (laminin substrate) there is sufficient stimulation to produce neurites, albeit small ones, while in information "poor" environments (ornithine) neurite-formation requires GAP-43 (phosphorylated GAP-43). Interestingly, Bixby (Bixby, 1989) has demonstrated a requirement for activated PKC in laminin-stimulated neurite outgrowth of chick ciliary ganglion cells. In order to observe this effect, the concentration of laminin must be suboptimal, again suggesting that GAP-43 (a downstream target of PKC) may be one of several outgrowth mediators and that its presence is only required when extrinsic growth signals are weak.

If the phosphorylation of GAP-43 has an important role in growth cone behavior, as the previous experiments suggest, then this role may come late in neurite development. Using an antibody that specifically recognizes the phosphorylated form of GAP-43, Dent and Meiri (Dent and Meiri, 1992) found that the phosphoprotein only appears after neurites have formed, and is localized in the distal tips of growth cones. Downregulation of PKC by prolonged phorbol ester treatment induced a rapid collapse of the growth cone, implying that GAP-43 phosphorylation has an important role in the maintainence of growth cone morphology. The phosphoGAP-43-specific antibody that is crucial to the authors' interpretation of the experiment may not be able to detect very small amounts of phosphoGAP-43. In the presence of an active phosphatase, for

example, the transient production of phopshoGAP-43 would not be detected. The CaM-dependent serine/threonine phosphatase calcineurin is abundant in neurites (not growth cones) (Ferreira et al., 1993) and may be one of the phospahtases that dephosphorylates phosphoGAP-43 (Liu and Storm, 1990).

Shea *et al.* (Shea et al., 1991) have studied neuritogenesis in the neuroblastoma cell line NE115. These cells express high levels of GAP-43, but they do not form neurites unless they are induced to differentiate with agents like dibutyryl cAMP (dbcAMP), demonstrating that GAP-43's presence alone is not sufficient to generate neurites. When the differentiating agent was introduced in conjunction with antibodies to GAP-43 (delivered in a phospholipid vehicle) neuritogenesis was halted. The result was specific to GAP-43 antibodies as both preimmune serum and antibodies to neurofilament H (another growthcone protein present at high concentrations in the cell line) had no effect.

In summary, phosphorylated GAP-43 appears to modulate the capacity of a particular cell to extend neurites (Widmer and Caroni, 1993). While the phosphoprotein is not required for neurite outgrowth *per se* (Baetge and Hammang, 1991) it may required for *stable* neurites to be formed (Shea et al., 1991), particularly in certain cell lines. There may be a similar requirement for the protein when the substrate does not promote adhesion (laminin v. ornithine) (Aigner and Caroni, 1993). The accumulation of phosphorylated GAP-43 in the growth cone signals a new phase in growth cone behavior, namely cessation of outgrowth and increased adhesivity (Dent and Meiri, 1992). This transition may be a precursor to synapse formation, since phosphoGAP-43 accumulates in the distal tips of neurites as they enter the field of their synaptic targets (Meiri et al., 1991).

Neurotransmitter release and GAP-43

The literature concerning GAP-43 and neurotransmitter release is confusing. Because PKC has been associated with neurotransmitter release in a number of systems (Kikkawa and Nishizuka, 1986), several groups investigated whether or not a major PKC substrate in hippocampal and synaptosomal preparations, GAP-43, had a role in this process. ^{32}P-orthophosphate labeled hippocampal slices were subjected to conditions known to induce neurotransmitter release, for instance depolarization with 30mM K^+ or phorbol ester, and GAP-43 phosphorylation was monitored (Dekker et al., 1989b). Phosphorylation of GAP-43 increased under these conditions. In addition, antibodies to GAP-43 completely inhibit calcium-induced noradrenaline release in permeated synaptosomes (Dekker et al., 1989a). However, *phosphorylation* of GAP-43, *per se*, does **not** appear to be a required step in the release mechanism. The introduction of PKC inhibitors, before or during calcium-induced depolarization, failed to block neurotransmitter release but effectively blocked GAP-43 phosphorylation (Hens et al., 1993b). Ivins *et al.* have showed that in PC12 cells treated with antisense RNA to remove GAP-43, evoked release of dopamine by either KCl-evoked depolarization or A23187 (a calcium ionophore) is prevented (Ivins et al., 1993). Therefore GAP-43 may have a role in transmitter release independent of of its phosphorylation state. Ivins *et al.* suggest that the release of CaM by GAP-43 under conditions of increased calcium could stimulate CaM-dependent enzymes and precipitate release. However, it is unlikely that CaM is working immediately downstream of GAP-43 in the process, since CaM inhibitors do not effect evoked release in PC12 cells (Matthies et al., 1988). On the other hand microinjection of anti-CaM antibodies, like anti-GAP-43 antibodies, blocks release of catecholamine in chromaffin cells (Kenigsberg and

Trifaro, 1985). A final interpretation of GAP-43 function in neurotransmitter release awaits a more precisely defined activity for GAP-43.

Synaptic plasticity revisited

In the years since the initial characterization of the correlation between GAP-43 phosphorylation and LTP, several groups have added important information to the story. Gianotti *et al*, (Gianotti et al., 1992) have repeated and confirmed the LTP experiment of Routtenberg *et al.* (Routtenberg, 1985)*in vivo* with oxygenated, ^{32}P-orthophosphate loaded, hippocampal slices. They elegantly demonstrate that while stimulation associated with LTP always gave increased GAP-43 phosphorylation, stimulation that failed to give rise to LTP was not associated with increased F1 phosphorylation, suggesting a causal relationship. Alternatively, the data may simply reflect a requirement for presynaptic PKC activation in LTP.

Another form of synaptic plasticity, called reactive synaptogenesis or collateral sprouting, has been investigated in the context of GAP-43 synthesis and phosphorylation. In adult rats the entorhinal cortex projects a large of fibers to the ipsilateral dentate gyrus and a smaller track of axons to the contralateral dentate gyrus. Lesioning of the cortex results in the axon terminals of the ipsilateral and contralateral dentate gyrus to "sprout" new terminals and form new synaptic contacts within the portion of the denervated section of the hippocampus. Lin *et al.* (Lin et al., 1992) found a rapid increase in the phosphorylation state of the GAP-43 already present in the hippocampus and a two-fold increase in the rate of transport of newly synthesized GAP-43 into the molecular layer. Again, GAP-43, and in particular the phosphorylation of GAP-43, is found to be associated with a process of "growth" and subsequent synaptogenesis.

DiLuca *et al.* administered methylazoxymethanol acetate (MAM) to pregnant rats during a specific gestational period in order to damage dividing neuroblasts in the hippocampus (Di Luca et al., 1993). The offspring of the rats were then tested for cognitive impairment. MAM-treated animals showed no capacity to learn. Hippocampal synaptic membranes were isolated and a *post hoc* phosphorylation assay was performed to determine the basal PKC activity. GAP-43 phosphorylation was drastically reduced compared to controls. While the investigators suspected that the reason for this decrease could have resulted from a cell loss in the hippocampus following MAM-treatment, their experiments revealed an essentially normal hippocampal formation with control levels of GAP-43. The learning impairment was correlated with a phosphorylation impairment, and not a gross structural deformation.

Since GAP-43 is a presynaptic protein, alterations in phosphorylation following LTP would have to be communicated by a retrograde messenger from the activated postsynaptic cell. A primary candidate for such a signal is arachidonic acid (AA), whose concentration rises sharply following activation of postsynaptic NMDA receptor, a crucial step in LTP induction (Dumuis et al., 1988). AA is a fatty acid and a known activator of some species of PKC (Shinomura et al., 1991). A recent study (Schaechter and Benowitz, 1993) related the presence of physiological concentration of AA to heightened GAP-43 phosphorylation in synaptosomal membrane preparations from the rat cerebral cortex. AA-stimulated PKC activity exhibits a reduced dependency on calcium as a cofactor for activation. The calcium level required for GAP-43 phosphorylation was found to be close to the calculated concentration of calcium following presynaptic activation, which can soar to 300μM in discrete microdomains near the plasma membrane (Llinas et al., 1992). Therefore, in the

presence of AA, derived from an activated postsynaptic cell, presynaptic PKC activation would be sufficient to generate phosphorylated GAP-43.

Membrane -binding of GAP-43

The enrichment of GAP-43 in growth cone membranes raises the question of how the protein is targeted to this specific region. The physical association of GAP-43 with particulate membrane fractions is resistant to high salt and is not dependent on calcium (Skene and Willard, 1981b). Nonionic detergent removes GAP-43 from the cytoskeletal elements, suggesting that the protein is tightly bound to the membrane (Meiri and Gordon-Weeks, 1990). Because GAP-43 lacks an obvious membrane-binding domain, and has few hydrophobic amino acids, Skene and Virag (Skene and Virag, 1989) investigated the possibility that a post-translational acylation of GAP-43 may be involved in membrane binding. Pulse-chase experiments showed that following synthesis, GAP-43 was cytosolic, but within twenty mintutes the major pool of GAP-43 became associated with the membrane fraction. Culturing rat embryonic cortical cells with labeled fatty acids revealed the specific acylation of GAP-43 with palmitate. Since this addition is invariably though a cysteine thioester-linkage (Sefton and Buss, 1987), the location of the palmitoylation was immediately mapped to the two sole cysteines at positions 3 and 4 on GAP-43 (Basi et al., 1987). Zuber *et al.* (Zuber et al., 1989) determined that the first ten amino acids of GAP-43 were sufficient to target a fusion protein to growth cones in transfected PC12 cells. This result was challenged by Liu *et al.* (Liu et al., 1991) who showed that although the first 10 amino acids of GAP-43 were required for targeting, they were not sufficient. They also showed that site-directed mutagenesis of the cysteines to alanine prevented palmitoylation (Chapman et al., 1992), and subsequently, prevented growth cone targeting. Zuber *et al.* make the important point that acylation *per se*

is probably not solely responsible for the distribution of GAP-43, since the staining is punctate, not uniform, and suggest a receptor for palmitoylated GAP-43 may ultimately direct its localization.

Dubious interactions...

The group lead by Gispen found that ACTH affected the overall levels of inositol phosphates in the brain (Jolles et al., 1980). When ACTH, albeit in concentrations three orders of magnitude greater than *in vivo* levels, is added to an *in vitro* membrane fraction, the phosphorylation of GAP-43 was inhibited. The inhibition of GAP-43 phosphorylation correlated with a stimulation in the production of phosphatidylinositol 4,5-bisphophate (PIP_2). Antibodies to GAP-43 immunoprecipitated a set of enzymes that they identified as PKC and PIP kinase. In an attempt to clarify the role of phosphoGAP-43 and PIPkinase, the proteins were semi-purified (Van Dongen et al., 1985) and an *in vitro* PIP kinase assay was performed in the presence of phosphoGAP-43 or dephosphoGAP-43. The authors present data that show a 40% reduction in PIP kinase activity when phosphoGAP-43 was present, and a 16% reduction in activity in the presence of dephosphoGAP-43. This was the last report concerning the two "interacting" enzymes.

A role for GAP-43 in the metabolism of the heterotrimeric GTP-binding proteins has recently been proposed by Strittmatter *et al.* (Strittmatter et al., 1990). G_o is a major growth cone protein. Attempts were made to identify interactions between this protein and GAP-43, another major growth cone protein. The proteins do not co-immunoprecipitate and GAP-43 is not retained on a G_o-chromatography column. However, GAP-43 was able to stimulate the binding of GTPγS to G_o. Further studies by this group indicated the effect was mediated by the first twenty-five amino acids of GAP-43, although the concentration needed to simulate the full-length protein were substantially higher. The effect was also

abolished when palmitoylated peptides were used in the assay(Sudo et al., 1992). This is problematic since the majority of GAP-43 is palmitoylated *in vivo* (Skene and Virag, 1989). Since the molar excess of GAP-43 needed to produce a small effect on the GTPase activity of G_o is at least 200-fold, the *in vivo* significance of the effect is cast in doubt.

While GAP-43 may have an important role in many processes, it status has been tarnished by the elusiveness of determining its function. It is now relegated to being simply a molecular *marker* for regeneration or a *marker* for LTP. This thesis is an attempt to develop a model system for studying GAP-43 wherein its function can be directly investigated.

CHAPTER II: Cloning of *nerd*

Introduction

Invertebrate and vertebrate lineages have been separated in time for over half a billion years (Buchsbaum et al., 1987). Several considerations must be made before the rational design of experiments to isolate related gene products is made. When one sets out to isolate a gene, particularly using DNA hybridization techniques, one rarely comes up without at least a false positive, particularly if one is willing to let the stringency level fall to very low levels. Once a "related" gene is found criteria must be established to determine whether one has isolated a "homolog" or a gene with some degree of relatedness. This point is not merely semantic since a primary goal of many *Drosophila* investigations is not only the elucidation of fly protein function, but by extension, the determination of vertebrate protein function.

The vast distance between *Drosophila* and mammals has afforded evolution a great opportunity to eliminate residues whose presence is less than critical. A comparison between homologous vertebrate and invertebrate molecules can often allow one to point out with some confidence the critical domains of a protein. The fact that calmodulin is 98% conserved between *Drosophila* and man (Yamanaka et al., 1987) suggests that most of the residues are needed for the activity of this ubiquitous calcium-binding protein. On the other hand, some proteins like cdc25 kinase (Alphey et al., 1992) can tolerate up to 70% of their amino acids being altered and still functionally substitute for their distant relatives *in vivo*. Functional rescue is very strong evidence in favor of two proteins being "homologous", irrespective of the degree of primary amino acid sequence similarity.

Many proteins have evolved a modular nature through "exon shuffling." The "universe of exons" that forms the basis of all extant proteins is thought to be

rather small, somewhere on the order of five to six thousand (Dorit et al., 1990). These modules can often function independently within a protein. For example, SH2 domains, which bind phosphotyrosyl residues , can be linked with phosphatases or kinases. Such an evolutionary"chimera" can target an enzyme to an environment where its activity is useful to the cell. Functional modules are usually conserved between true homologs, though their relative position within the protein can vary.

A comparison between several vertebrate GAP-43 homologs reveals the conservation of only the N-terminal quarter of the protein through the last 200 million years. LaBate and Skene (LaBate and Pate Skene, 1989) isolated a goldfish protein with DNA homology to a rat GAP-43 cDNA using low stringency hybridization conditions. The protein is a homolog based upon a conserved linear amino acid sequence and the GAP-43-like feature of enhanced expression of the protein during experimentally-induced regeneration of the goldfish optic nerve. They couple their findings to the negative result of not having found a more obviously related protein.

The sequence of the fish cDNA they isolated (Figure II.1) shows a high sequence homology to mammalian GAP-43, 84% identity and 91% similarity, within the first 57 residues. This conservation evaporates very quickly thereafter, falling off to less than 10%. The overall amino acid identity between the rat and fish molecules is about 25%. Although the primary sequence has been altered, several biophysical features have been retained, including the length of the proteins and the highly-charged, alanine-enriched amino acid composition. Hydrophobic residues are rare and aromatic residues are totally absent. These features combine to give GAP-43 two of its hallmark features, namely its very high solubility, even in 2.5% perchloric

Figure II.1: Vertebrate GAP-43 proteins have diverged rapidly

Sequence comparison between goldfish GAP-43 (LaBate and Pate Skene, 1989) and rat GAP-43 (Karns et al., 1987) using the FASTA alignment program (Pearson and Lipman, 1988). Note that following amino acid 56, there is a marked breakdown of amino acid identities between the two proteins. The underlined sequences are the two cysteines at position three and four which are palmitoylated *in vivo*, and the calmodulin-binding/PKC-site module (GAP43 module). Abbreviations for the amino acids as they appear throughout this study are as follows: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamate; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

VERTEBRATE GAP-43s ARE HIGHLY DIVERGENT

```
fishGAP43 MLCCIRRTKPVEKNEEADQEIKQDGTKPEENAHKAATKIQASFRGHITRKKMKDEDKDGE
rat GAP43 MLCCMRRTKQVEKNDE-DQKIEQDGVKPEDKAHKAATKIQASFRGHITRKKLKDEKKGDA
CONSENSUS MLCC.RRTK.VEKN.E.DQ.I.QDG.KPE..AHKAATKIQASFRGHITRKK.KDE.K...

fishGAP43 NDTAPDESAETEEKEERVSPSEEKPVEVSTETAEESKPAEQPNSPAAEAPPTAATDSAPS
rat GAP43 PAAEAEAKEKDDAPVADGVEKKEGDGSATTDAAPATSPKAEEPSKAGDAPSEEKKGEGDA
          .....................E......T..A....P.....S.A..AP.........

fishGAP43 DTPT-KEEAQEQLQDAEEPKETENTAAADDITTQKEEEKEEEEEEEEEEEEAKRADVPDDT
rat GAP43 APSEEKAGSAETESAAKATTDNSPSSKAEDGPA-KEEPKQADVPAAVTDAAATTPAAEDAA
          .....K....E....A..........A.D....KEE.K...........A......D..

fishGAP43 ----PAATESQETDQTDK-KEALDDSKPAEEAGKDQNV
rat GAP43 KAAQPP-TETAESSQAEEEKEAVDEAKPKESARQDEGKEDPEADQ
          ....P..TE..E.......KEA.D..KP.E.A..D..
```

acid, and its extended rod-like shape, which may account for its unusual retarded migration on polyacrylamide gels (Masure et al., 1986b). Based on the amino acid composition, GAP-43 is only 25kD. However, on SDS-PAGE gels, GAP-43's apparent molecular weight is 43kD, hence its name. P-57 (Wakim et al., 1987), pp46 (Meiri et al., 1986) and B-50 (Gispen et al., 1985) all describe the same protein with misleading molecular sizes on gels.

Since most modern molecular biology screening techniques require that homologous proteins share protein sequence, for immunological screening, or DNA homology, for hybridization or PCR screeening, it is probably fair to say that had the goldfish and rat GAP-43 proteins not had the first 60 amino acids in common, the identification of the fish protein would have been much more difficult. Growth-associated neuronal proteins could have been purified and sequenced, but this would have taken a much longer time and demanded a highly involved, systematic approach. If the C-terminal, nonconserved region of GAP-43 lends a crucial function to GAP-43 then the amino acid sequence that determines this function is very flexible. It has been suggested (LaBate and Pate Skene, 1989) that a "neurofilament-like" structure exists in the C-terminal domain and may give GAP-43 the ability to associate with cytoskeleton. Indeed, reports have suggested a cortical cytoskeleton association (Meiri and Gordon-Weeks, 1990) but this property has yet to be mapped to a region of the protein. Moreover, the physical association of GAP-43 with cytoskeletal elements has only been crudely determined by immunofluorescence and co-sedimentation of GAP-43 with the membrane fraction (Moss et al., 1990). One report (Hens et al., 1993a) included an actin-binding study, but this research indicated that neither GAP-43 or phospho-GAP-43 affected actin polymerization or filament organization.

One compelling argument that there is almost no constraint on the sequence of GAP-43 following the first 60 amino acids is that all of the major "activities" of GAP-43 are conspicuously localized in the conserved N-terminal residues. As reviewed in Chapter I, GAP-43 is a neuronal, membrane-associated, calmodulin-binding, PKC substrate. Neuronal membrane targetting is conferred in part by the first 10 amino acids, which contain two cysteines which are palmitoylated *in vivo* (Skene and Virag, 1989). The calmodulin-binding peptide has been mapped to amino acids 38 to 52 (Alexander et al., 1988). The single *in vivo* PKC site has been mapped to serine 41 (Au et al., 1989). Therefore all of the well-documented biochemical activities of GAP-43 fall involve the conserved domain. This region may be considered a module.

Recently another neuron-specific mammalian protein has come under scrutiny that bears some similarity to GAP-43. This protein, neurogranin, binds calmodulin with a similar calcium-sensitivity as GAP-43, is phosphorylated by PKC on a similar serine, and binds phospholipids like GAP-43 (Baudier et al., 1991; Coggins et al., 1993b). Neurogranin is localized to dendritic spines and neuronal soma (Represa et al., 1990), contrasting the primarily presynaptic localization of GAP-43 (Gispen et al., 1985). This distinction seems to be the major difference between two proteins. The only primary amino acid sequence similarity between the two proteins is confined to a region which I will refer to as the GAP43 module (Figure II.2) and has been shown to confer the calmodulin-binding activity and harbor the seryl residue that is a substrate of PKC. The GAP43 module is also responsible for phospholipid association (Houbre et al., 1991). It has been suggested that GAP-43 and neurogranin play similar roles, one presynpatically and one postsynaptically.

Clearly, the GAP43 module is an integral domain in GAP-43 and neurogranin. A *Drosophila* homolog should maintain the primary sequence of

Figure II.2: GAP-43 and neurogranin are related in sequence through the GAP43 module only

Sequence comparison between murine neurogranin (NG) and rat GAP-43. Bold letters indicate an amino acid identity between the two proteins. The consensus defining the GAP43 module is underlined.

GAP-43 MLCCMRRTKQVEKNDEDQKIEQ**D**GVKPEDK**A**HK**AA**T**KIQASFRGH**ITR**KKL**K**DEKK
NG MDCCTESACSKPDDDIL**D**IPLDDPG**A**NA**AA**A**KIQASFRGH**MA**RKK**IK**SGEC
AA-KIQASFRGH--RKK-K
GAP43 module

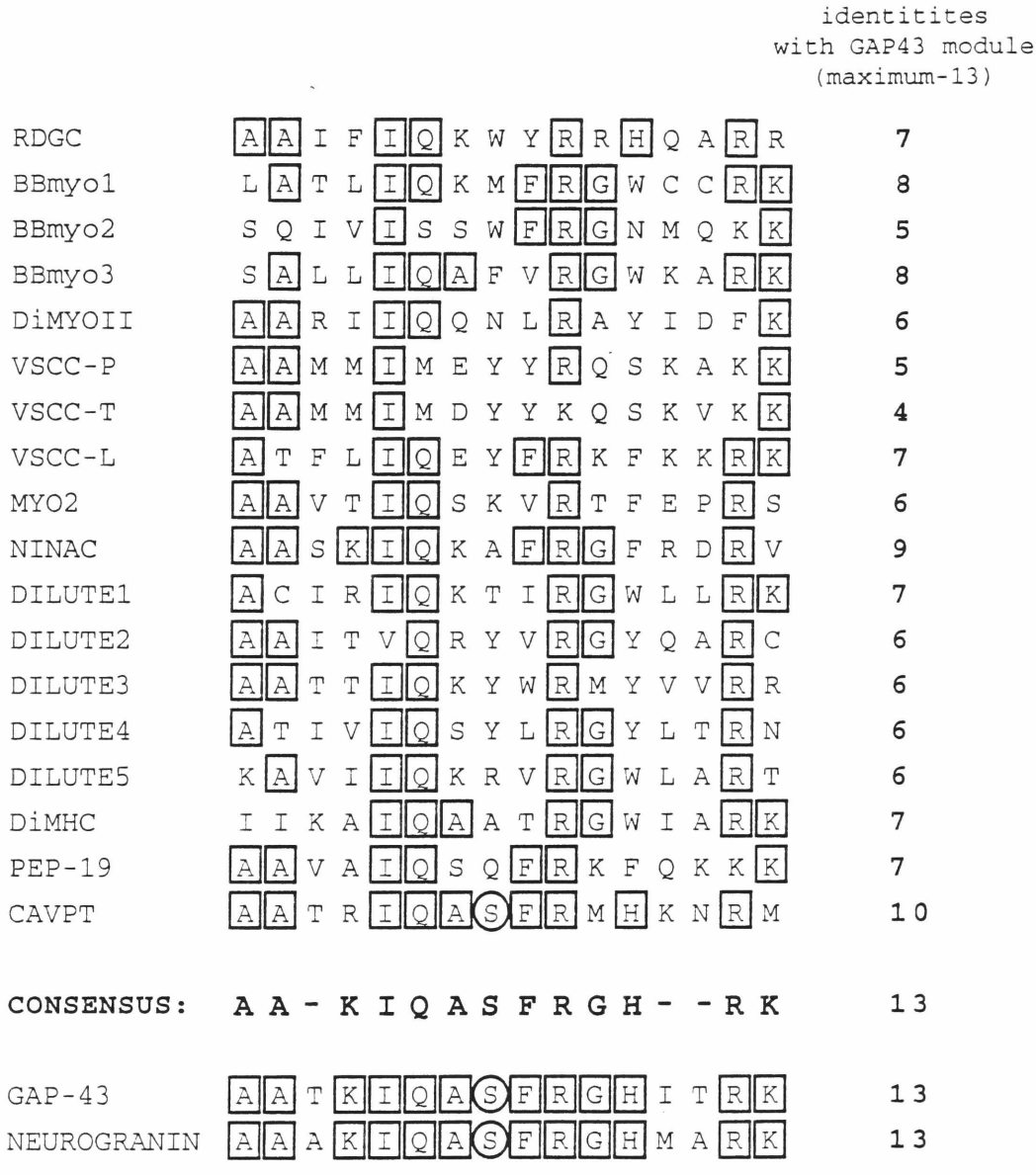
GAP-43 **G**DAPAAEAEAKEKDD**A**PVAD**G**VEKKEGDGSATTDAAPATSPKAEEPSKAGDAPSEE
NG **G**RKGPGPGGPGGAGG**A**RGGA**G**GGPSGD

GAP-43 KKGEGDAAPSEEKAGSAETESAAKATTDNSPSSKAEDGPAKEEPKQADVPAAVTDA

GAP-43 AATTPAAEDAAKAAQPPTETAESSQAEEEKEAVDEAKPKESARQDEGKEDPEADQ

Figure II.3: GAP-43 and neurogranin belong to the 'IQ' motif family of proteins

Assorted 'IQ' motif proteins identified in a low stringency FASTA search with the GAP43 module against the GENBANK database. A comparison with the canonical 13-residue GAP43 module consensus appears to the right of each entry and is expressed as the number of identities. Abbreviations are as follows: DiMYOII, *Dictyostelium* myosin II (Delozanne and Spudich, 1987); RDGC, *retinal degeneractionC* of *Drosophila* (Steele et al., 1992); BBmyo1-3, brush border myosin I, repeat 1,2, or 3 (Swanljung-Collins and Collins, 1992);p190, chicken brain p190 (Espreafico et al., 1991); VSCC-P,T and L, voltage-sensitive calcium channels, N-,P-,L- and T-subtypes (Fujita et al., 1993; Soong et al., 1993); MYO2,*Saccharomyces* MYO2 (Johnston et al., 1991); NINAC, *Drosophila ninaC* gene product (Montell and Rubin, 1988); DILUTE1-5, murine *Dilute* gene product, repeats 1-5 (Mercer et al., 1991); DiMHC, *Dictyostelium* myosin heavy chain (Cheney and Mooseker, 1992); PEP-19, cerebellar protein PEP-19 (Sangameswaran et al., 1989); CAVPT, calcium vector protein target protein in *Amphioxus* muscle (Takagi and Cox, 1990).


identitites
with GAP43 module
(maximum-13)
RDGC A A I F I Q K W Y R R H Q A R R 7
BBmyo1 L A T L I Q K M F R G W C C R K 8
BBmyo2 S Q I V I S S W F R G N M Q K K 5
BBmyo3 S A L L I Q A F V R G W K A R K 8
DiMYOII A A R I I Q Q N L R A Y I D F K 6
VSCC-P A A M M I M E Y Y R Q S K A K K 5
VSCC-T A A M M I M D Y Y K Q S K V K K 4
VSCC-L A T F L I Q E Y F R K F K K R K 7
MYO2 A A V T I Q S K V R T F E P R S 6
NINAC A A S K I Q K A F R G F R D R V 9
DILUTE1 A C I R I Q K T I R G W L L R K 7
DILUTE2 A A I T V Q R Y V R G Y Q A R C 6
DILUTE3 A A T T I Q K Y W R M Y V V R R 6
DILUTE4 A T I V I Q S Y L R G Y L T R N 6
DILUTE5 K A V I I Q K R V R G W L A R T 6
DiMHC I I K A I Q A A T R G W I A R K 7
PEP-19 A A V A I Q S Q F R K F Q K K K 7
CAVPT A A T R I Q A S F R M H K N R M 10
CONSENSUS: A A - K I Q A S F R G H - - R K 13
GAP-43 A A T K I Q A S F R G H I T R K 13
NEUROGRANIN A A A K I Q A S F R G H M A R K 13

this region. The GAP43 module is part of the 'IQ' motif family (Cheney and Mooseker, 1992; see Figure II.3). The 'IQ' module represents a region participating in protein-protein interactions, often binding calmodulin or calmodulin-like polypeptides. For example, the NINAC protein is believed to participate in the targeting of calmodulin to microvillar membrane in the *Drosophila* retina (Porter et al., 1993) via its 'IQ' module. A similar role for GAP-43 has been postulated by Storm's group (Andreasen et al., 1983) but never demonstrated. Calcium vector target protein (Takagi and Cox, 1990) has an 'IQ' domain very similar to GAP-43 and neurogranin but does not bind calmodulin. Instead, its binds calcium vector, a calcium-binding protein with homology to calmodulin.

The absolute conservation of residues in the GAP43 module between fish and man implies that the module may have a novel, sequence-dependent function which none of the less-related 'IQ' proteins has. Given the rapid divergence of homologous GAP-43 proteins outside of the module and the similarity in function between GAP-43 and neurogranin (which share only the GAP43 module sequence in common) attention was focused on finding a *Drosophila* protein with the GAP43 module. The premise was that at the very least a fly gene with GAP-43-like functions would be uncovered.

Previously, Ng *et al.* had reported the sequence of a fruitfly cDNA that they isolated through its DNA homology to a full length rat GAP-43 cDNA (Ng et al., 1989). The gene is called *kz30*. Apart from the fact that the RNA transcripts do not localize to neurons *in vivo* (a serious problem in itself) the theoretical translation of the KZ30 cDNA does not yield a product with any homology to the conserved region of GAP-43. The protein does not have a recognizable GAP43 module or an 'IQ' domain. It is therefore very unlikley that KZ30 is a GAP-43

homolog. The original authors have abandoned the idea that there is a functional connection between GAP-43 and KZ30 (Eberl et al., 1992).

Results

To extend the search for a *Drosophila* homolog of GAP-43, I focused on the amino-terminal domain of rat GAP-43 as the sequence most likely to have been maintained if the gene were conserved in very distantly related phylum. For the screen, oligonucleotide primers were synthesized for PCR amplification of a cloned rat cDNA (Karns et al., 1987). The 5' primer corresponded to the conserved palmitoylation site and the 3' primer included sequences encoding the GAP43 module. The resulting 161bp PCR fragment was hybridized at low stringency with a genomic DNA library. A genomic library was used in order to avoid biased expectations concerning the level of expression or the developmental timing of expression of any putative GAP-43 homolog. A phage, called B3, was isolated and a hybridizing 2kb SacI subclone from B3 was sequenced. The conceptual translation of this sequence showed a near-perfect homology to amino acids 33 to 50 of GAP-43, the GAP43 module. As mentioned above, this region includes the single *in vivo* PKC phosphorylation site and the calmodulin-binding domain of GAP-43 and is absolutely conserved in all vertebrate GAP-43 molecules, as well as neurogranin.

Two types of cDNAs were repeatedly isolated from a pupal library screened with the genomic fragment, a 1.9kb species (clone pZd10) and a 2.5kb species (clone pZd6). Both were sequenced and found to contain identical 3' ends but different 5' ends (Figure II.4). A Northern analysis was performed on embryonic and adult head RNA in order to verify that the two cDNAs represented distinct, mature messages and not partially processed cDNAs. A major 1.9kb poly(A)+ RNA, and a minor 2.5kb poly(A)+RNA are present in late

Figure II.4: Nucleotide sequence of *nerd* cDNAs

Nucleotide sequence of full length cDNAs for *nerdL* (pZd6) and *nerdS* (pZd10). The numbering above the amino acids is based on a conceptual translation from the first methionine in an appropriate context for initiation in *Drosophila.* (Cavener, 1987). Amino acid 119 of NERD-L corresponds to amino acid 1 of NERD-S. The arrowhead indicates where the two cDNAs begin to overlap. The 5'-noncoding sequence of pZd10 is listed below the pZd6 sequence and is also marked with an arrowhead. The underlined sequences are the three GAP43 modules discussed in the text. Double-underlined sequence is a consensus myristoylation site.

nerd-L 1 cagtagttgtgcctg
agcttgtgccagagacagtcagtttcgctgcgagtctgccatccagctggtttccgcttccggttccgtgcgagttctttg
gctagtaaattgtggttgtgcacctttttctaatgacttttgtaaacaccaaagttttttgaacaaaaaaaattaccgagg
cacaacgtatttttgtaaacatttaatgcgaaaaaggtttcagctcgcgaaagtcaaagtgcaaattggccagaaacaaca
agtatcgtttataaactagtttccagttcggatgtgtgtgtgtgtactatagtgtgtgtgtgtgcaattgattaaacaaca
caaggtaacctaggccaactggaagcgtagagactccatcaaaagccgcggactaaattgccaaatcctcgatccaataag

1
ATGGGCTGCAACACCAGCCAGGAGCTAAAGACGAAGGATGGCGCAGCCATGGATGCGGTCAGCAACGGCGAACCGGAGCCC
M G C N T S Q E L K T K D G A A M D A V S N G E P E P

28
AGTGCTCCTCCCTTGGAGGGCGAGTCATCCAAGTCATCCGCTAGCAATCATACGAATCACGCCAAATCCAGTTCAATTATC
S A P P L E G E S S K S S A S N H T N H A K S S S I I

55
TCCAACGGCGAGGCGAAGGCGGCCAACGGGGGCGGCGCAGTTGGCGGGGGGAGTGGCAAGTCGGAAGCAACAAATGGCATT
S N G E A K A A N G G G A V G G G S G K S E A T N G I

82 ▼
GATCGTCCCTGCGACAAGGCGGCAATCACGGAGTTCAATGACGACGAGGACGAAGCAAAAGCCGCAACAAAAATCCAAGCC
D R P C D K A A I T E F N D D E D E A K A A T K I Q A

109 119/1
GTCTTTCGAGGCCACAAAGTGAGAGAAACCATGAAAAAATCGGAAACCAAAACTGCGACCAACAACGGCAGTGCCGCCGGA
V F R G H K V R E T M K K S E T K T A T N N G S A A G

136/18
GCAGCTCCATCGGCAGCAGCCGCCGAGGCAGCCGCATCCGCAGAGCCAACCAAAGCCGAGCTAGAGGCTGAATTCGATCCC
A A P S A A A A A E E A A A S A E P T K A E L E A E F D P

163/45
AACGACAAGGATCTGTGCCATGCTGCATTGAAGATTCAGTCCACTTTCCGTGGTCATTTGGCACGCAAGCTGGTGAACAAG
N D K D L C H A A L K I Q S T F R G H L A R K L V N K

190/72
GATGCGCCCGAGGACGAGGACATTCAGGAGATAACCAAGAAGGTGGCCGAGGAGTTGGACATAGATTTAACCGATCCCGAG
D A P E D E D I Q E I T K K V A E E L D I D L T D P E

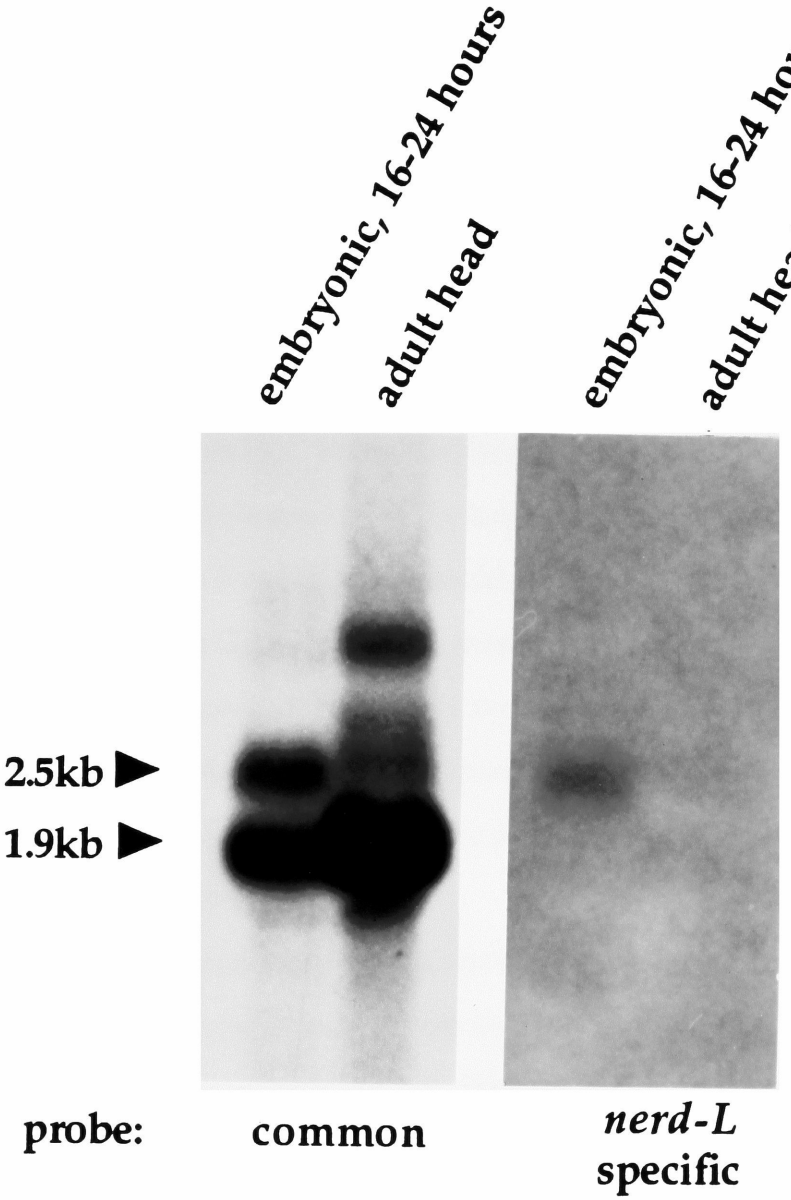
217/99 240/122
CTTAACAAGGCTGCTACCAAAATTCAGGCCTCGTTCCGGGGCCACAAGACCCGCAAGGATGCCAATCCCGAGTAAggagct
L N K A A T K I Q A S F R G H K T R K D A N P E *

caccacttgaattttcattgccctcggtgggctagtcacagatgatgaagctaacttaatgcttacgcaagtgaataaaat
tattggtaaattgtaaatgtgtaaaaggccaatcgagatgagggcacacaattgtaaacttttagtgaatatgctttagc
cggcatggcgaacccaaatgattctcataattattaaataaattaagtcgagggcaaagttacatttagttggcaaagctt
taataaatgaatagtaaaaccacattatgcacatgatgattcggagttacgcgacatttacagatacattattgcggcttg
tcttgttctattacatacccttagagtcgtaaattaataaattggtattagttaatatttaattatattacatttcgtttt
tggactttgctattaaacccaagaccgtaactaaaccaaaaataaaagtgaactaacgttaaattgtcaaagaaaaacctа
atgttacaaaataataaataaaaggcacgatgttccataaactttattgtagggaaatgagaaaccaaacaaaagacaaat
gcaaggaaactgctgaacaaatttttgggaaatcatttccatttgtcgggccaatgccaaaagtaaaaaattaattgtac
ctacgagcgtatgaatatgaattagaaaccaaaggaactcaatgttgtttcaaattaaggcaaattaggggtacccaagca
acatgatgcagcttcgagtcgtcaactcaacaatttttcagccctcgggctctatataaactgaaaagtcaatgaacatga
agataactaatctggtggcacaattttcgtttttctaccaagtgtattacaatatttgtctacttaacaattgatcctcga
gtgggaaactgaaatacacacatacatatatatatacctatacagatgcaaagacagatattagaccgagttgaattggaa
aatatttttttttcggccacagctgagaaaattgttaccatgtggcaacggaagccaataatgaaatatttaaaattcaaat
caattaactacaaagaattaataacaagctaatgaacataattgaaattatattagagatcaaagtcaaattcaaaataaa
ttgcataaaattattacatttaaaaagcaaatgggtaaaaccaattctatgtggataaatgaaaaggaaaaaccagaatat
gaaagcaaaccatatacaatcctaacaaacaaagaatgtcccaactaaattttaattgagtaataaaaataaatacaataa
agatgcaaaaaaaaaaaaaaaaaa 2470/1922

▼
nerd-S 1 ggcacgaggaagcgttcggttcgataggttgccccaaagagagaaagtccaagcggaagtcctttgagccc
agcaaaagggaaagtgcgcgcgaatttaaatattaatagaataaaacaaactatcggtttgtacgtgttttgtgctgagcc
tttctgcagaagagccagagcca(agcaaaa...)

Figure II.5: GAP-43-related fly gene produces two transcripts that are present in the RNA from late embryos and adult heads

Ten micrograms of total RNA from either 16-24 hour embryos or adult head capsules was run on a 1% formaldehyde gel and blotted to Nytran membrane. In the left panel, a 452 bp ^{32}P-αGTP labeled riboprobe from the 3'-terminus of the *nerdL* cDNA was hybridized to the membrane. In embryonic RNA two prominent bands were visualized, a 2.5kb and a 1.9kb band. In adult head RNA the 1.9kb fragment predominated. The large band in this lane (>4kb) is never seen in poly(A)+ selected RNA (see Figure III.1) and is assumed to be an unprocessed transcript. In the right panel, a 460bp double-stranded PCR product specific to *nerdL* was nick-translated with ^{32}P-αdCTP and hybridized with the same filter as in the left panel. Only the larger 2.5kb trnascript is seen in late embryonic RNA. It is apparently absent in head RNA although using PCR the larger transcript is detectable. This figure demonstrates that the two isolated cDNAs represent alternatively spliced transcripts with a common 3' end and different 5' ends. A probe specific for the smaller cDNA detected the 1.9kb message in both lanes (data not shown).


embryonic, 16-24 hours
adult head
embryonic, 16-24 hours
adult head
2.5kb
1.9kb
probe:
common
nerd-L
specific

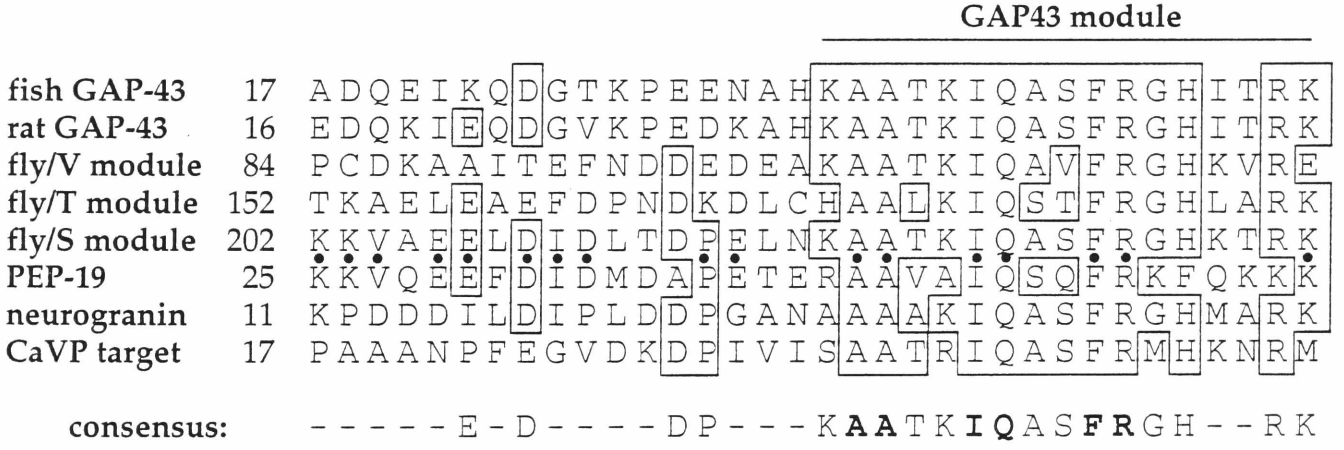
embryonic RNA (Figure II.5). The larger transcript is not detectable in adult flies at this level of analysis but is detectable by the very sensitive technique of PCR (data not shown).

The larger cDNA has an open reading frame encoding a 240 amino acid protein with predicted M_r of 24kDa. The last 122 amino acids of this protein constitute the entire open reading frame of the smaller cDNA and yield a protein with a predicted M_r of 13kDa. The region of overlap between the two protein isoforms contains the region of homology to GAP-43 uncovered in the genomic clone B3. In fact, three repeats of the 17-residue GAP43 module sequence are present in the larger protein and two copies are present in the smaller (Figure II.6). I denote the modules V, T and S for the amino acid code of the residue in the position of the putative PKC phosphorylation site. The nucleotide sequence encoding the GAP43 module specific to the larger isoform (V-module) is contained within the smaller cDNA but would not be translated in the smaller protein. Outside of these GAP43 modules there is no obvious homology to GAP-43 or neurogranin. Because the fly proteins are composed of multiple GAP43 modules, I have named the gene *nerd*, for neuromodulin-related domains. The two putative protein products are referred to as NERD-L, for the long isoform, and NERD-S, for the short isoform.

Although I could not detect significant homology between mammalian GAP-43 and the NERD proteins outside the GAP43 module, the overall amino acid composition of the proteins is similar. This is reminiscent of the conservation of amino acids in the C-termini of rat and goldfish GAP-43. The proteins are rich in alanine (GAP-43, 20.2%; NERD-S, 19.5%; NERD-L, 17.4%), they contain many charged amino acids (GAP-43, 40%; NERD-S, 33%; NERD-L,30%), they have few hydrophobic residues and are free of tyrosine and tryptophan. Database searches revealed an additional homology in the NERD

Figure II.6: The NERD proteins have two or three GAP43 modules

Alignment of the three GAP43 modules of NERD (fly/V-,T- and S-modules) with rat and goldfish GAP-43, as well as three other proteins with homology to the GAP43 module sequence. Boxed amino acids represent identity with the consensus. Residues absolutely conserved in all sequences are in bold face in the consensus. Additional identities between the fly S-module and PEP-19 are indicated with closed circles.


GAP43 module
fish GAP-43 17 ADQEIKQDGTKPEENAHKAATKIQASFRGHITRK
rat GAP-43 16 EDQKIEQDGVKPEDKAHKAATKIQASFRGHITRK
fly/V module 84 PCDKAAITEFNDDEDEAKAATKIQAVFRGHKVRE
fly/T module 152 TKAELEAEFDPNDKDLCHAALKIQSTFRGHLARK
fly/S module 202 KKVAEELDIDLTDPELNKAATKIQASFRGHKTRK
PEP-19 25 KKVQEEFDIDMDAPETERAAVAIQSQFRKFQKKK
neurogranin 11 KPDDDILDIPLDDPGANAAAKIQASFRGHMARK
CaVP target 17 PAAANPFEGVDKDPIVISAATRIQASFRMHKNRM
consensus: -----E-D----DP---KAATKIQASFRGH--RK

proteins to the developmentally regulated, rat cerebellar protein PEP-19 that overlaps a portion of the the S-module (Figure II.6). The function of PEP-19 is not known (Sangameswaran et al., 1989).

Discussion

In this chapter I have obtained evidence of a novel gene in *Drosophila* that encodes two geneproducts with homology to GAP-43 and neurogranin. The region of homology is precisely the domain that identifies GAP-43 and neurogranin as related proteins, the GAP43 module. Technically, it would be a mistake to call *nerd* a GAP-43 homolog, since at the protein level it is equally related to neurogranin and GAP-43. Other groups (Ng et al., 1989) have attempted to isolate a fly GAP-43 homolog. By several criteria they have failed to do so. The reason for their failure is probably two-fold. First, the stringency of hybridization was too high in the Ng experiment, 30% formamide was used instead of 20% as in my experiment. Also, they used a larger probe than I did and consequently increased the nonspecific background. At very low stringency conditions, it is crucial to limit the probe size to 'critical' regions only. In addition, the PCR probe in my screen contained degenerate primers for the GAP43 module, a fact which increased the likelihood of finding a distantly related sequence. Secondly, Ng *et al.* screened a 0-12 hour embryonic cDNA library, perhaps assuming that a GAP-43-related protein would be present early in development when neurite outgrowth is taking place (Hartenstein, 1988). As discussed later, the *nerd* gene is not expressed before 12 hours of development.

If GAP-43 and neurogranin define a small family of functionally related proteins, then one could speculate that an archetypal GAP43 module-containing gene must have existed at one time, before the invertebrate-vertebrate division, some 400 million years ago (Buchsbaum et al., 1987). GAP-43 and neurogranin

may have arisen by a gene duplication event. This episode may have enabled the vertebrate lineage to develop an additional neuronal function. A similar function may have been derived in the invertebrate lineage from the generation of different proteins by alternate splicing as in the *nerd* gene to produce NERD-L and NERD-S.

In the remaining chapters I explore the expression, biochemistry and genetics of the *nerd* gene products.

Chapter III: NERD expression

Introduction

Although GAP-43 has recently been found in reactive astrocytes (Deloulme et al., 1990) and muscle precursors in the the developing chick limb bud (Stocker et al., 1992), the protein is predominantly associated with neurons. Most if not all neurons express GAP-43 at some time during their development, and many maintain some level of GAP-43 expression in adult tissue (Neve et.al., 1988). As reviewed in Chapter I, GAP-43's neuronal expression is one basis for ascribing function to the molecule. For instance, the protein is postulated to have a role in axonal regeneration following injury (Skene, 1981). In addition, enhanced expression has been correlated with successful regeneration but not with failed regeneration.

In Chapter II, a molecular description of the NERD proteins was outlined. One modest, but crucial, requirement in claiming that NERD represents a homolog of GAP-43 is that the expression pattern of the gene includes nervous system tissue. If this were not to be the case, it would be difficult to argue that the proteins had homologous roles, though it would not rule out the possibility that they have similar functions in different tissues. A nervous system-specific expression of the *nerd* gene takes on an increased importance because the sequence of the NERD and GAP-43 proteins has diverged considerably. In this chapter, I analyse the temporal and spatial embryonic RNA distribution of *nerd* as well as the localization of the NERD protein. Based on the expression pattern, further arguments supporting the claim that NERD is a GAP-43 homolog are proffered. In addition, the temporal expression analysis is used to eliminate certain developmental roles for the fly protein and, by analogy, the vertebrate protein.

Results

A Northern was constructed to include RNA collections from several important developmental stages (S.Kidd, personal communication) and was probed with a subclone of the *nerd* gene encompassing 200bp of the 3' exon. This fragment should hybridize to both *nerdS* and *nerdL* transcripts. As seen in Figure III.1, both messages appear to be regulated temporally and spatially in a similar manner. However, the level of expression of *nerdS* is greater at all times. Although it is difficult to see any *nerdL* transcript in adults, PCR-analysis indicates that the larger transcript is present at very low levels (data not shown).

Expression from the *nerd* locus begins at 12 hours after egg-laying (AEL) and reaches a peak level in the last stages of embryogenesis, from 16 hours to 24 hours. The level of message gradually declines through the first and second larval instars and is almost absent during the last larval molt. Expression resumes during metamorphosis and persists into adulthood. Since *nerd* is expressed at relatively high levels in the adult brain (see Chapter II, Figure II.4), the apparent decrease in expression in adult females may be due in part to the proportional increase in non-nervous tissue to nervous tissue in females (*i.e.* increased fat body).

To determine the *in situ* localization of the *nerd* message, the same fragment used in the previous experiment was labeled with digoxigenin-dUTP and hybridized to fixed embryos (Figure III.2). In agreement with the Northern analysis, only late-stage embryos are labeled. From the number, position and size of the staining cells (Ghysen et al., 1986), all the labeling appears to be restricted to neurons, although some staining in the peripheral sensory structures may include non-neuronal accessory cells. Both the central (Figure III.2A,B) and peripheral (Figure III.2C-F) nervous system contain stained cells. The staining in the central nervous system (CNS) is bilaterally symmetric and metameric. At this

Figure III.1: Temporal expression pattern of *nerd* transcripts

Poly(A)$^+$ RNA (5 μg per lane), isolated at the indicated developmetal period, was probed with the 2kb SacI fragment of the genomic clone B3. This fragment contains 234bp corresponding to nucleotides 496 to 730 of the pZd6 cDNA sequence presented in Figure II.4. Two transcripts are detected, encoding *nerdL*(2.5kb) and *nerdS*(1.9kb). The identities of these transcripts were also confirmed using pZd6- and pZd10-specific probes (Figure II.5).

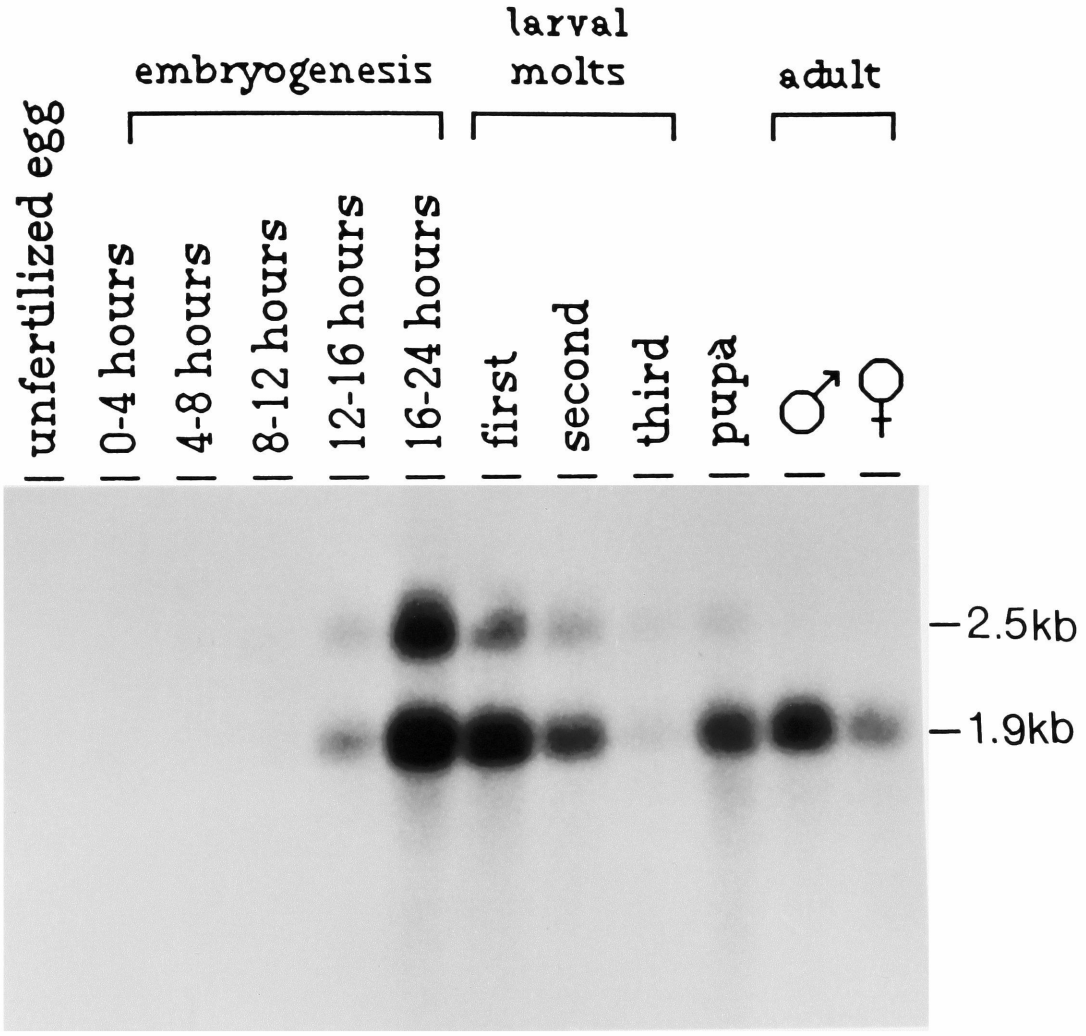
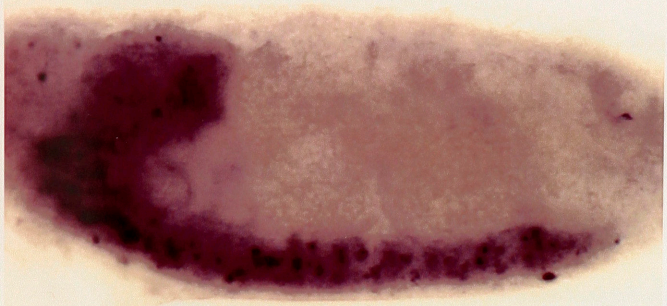
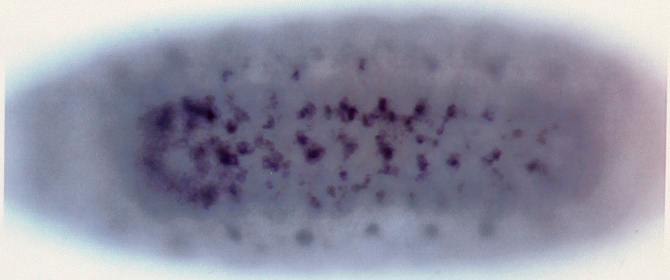
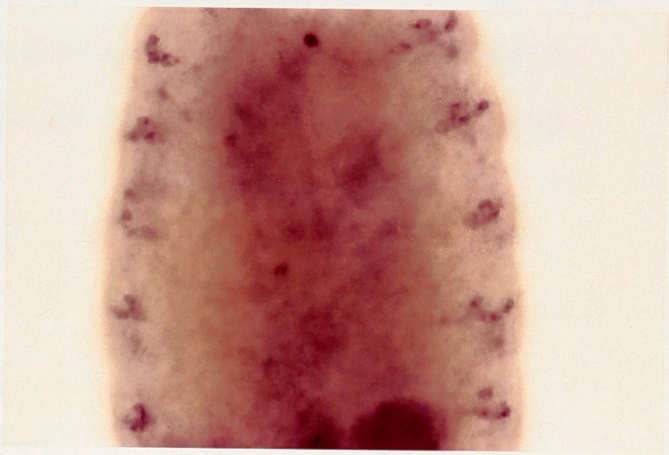

embryogenesis
larval molts
adult
unfertilized egg
0-4 hours
4-8 hours
8-12 hours
12-16 hours
16-24 hours
first
second
third
pupa
♂
♀
2.5kb
1.9kb

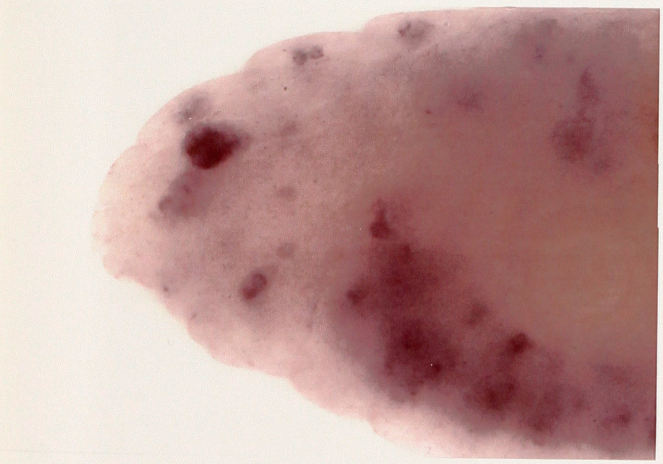
Figure III.2: *nerd* transcripts are found exclusively in the nervous system of the the developing embryo

A cDNA fragment corresponding to nucleotides 896 to 1388 was labeled with digoxigenin and hybridized to wild-type Oregon-R embryos reared at room temperature. Staining was detected only in embryos of age greater than 12 hours after egg-laying. The embryos represented here are approximately 16 hours old. Anterior is to the left in all panels except (D) where anterior is down and (F) where it is up. (A) Lateral view of the ventral nerve cord (vnc) and brain (br)showing widely distributed staining of neuronal cell bodies. In (B) a ventral view highlights small metameric clusters in the midline and lateral regions of the vnc. (C) A more superficial lateral view illustrating staining in the dorsal sensory structures (arrowheads). (D) Dorsal view of (C) showing groups of sensory neurons staining for *nerd* transcripts (arrowheads). (E) Higher magnification of the anterior sense organs, including the heavily stained antennomaxillary ganglion (amx). (F) A dorsal view of an embryo double-labeled with *nerd* digoxigenin probe (blue/black) and an antibody to horseradish peroxidase (light brown), which stains neuronal membranes. The cluster of neurons in the antennomaxillary ganglion (amx) contain *nerd* RNA, and the large nerve fascicle exiting posterior to the sense organ is the antennal nerve (nan).









stage of development there are too many neurons in the CNS to make an accurate determination as to which cells are stained, but within the peripheral nervous system (PNS) it appears that all of the sensory neurons contain *nerd* mRNA. The pattern of sense organs that make up the *Drosophila* PNS is highly stereotyped (Hartenstein, 1988). Dorsal, lateral and ventral groups of stained sensilla can be distinguished in thoracic and abdominal segments. In addition, the 20 cells that make up the antennomaxillary ganglion, a larval sense organ, are heavily stained, as demonstrated in Figure III.2E and F. In this figure digoxigenin labeled embryos were doubled labeled with anti-HRP antibodies, an procedure which stains most of the neuronal membranes in the embryo (Jan and Jan, 1982). The antennal nerve fascicle can clearly be seen exiting posteriorly to the grape-like cluster of darkly stained, *nerd*-containing neurons that make up this prominent larval sense organ.

Discussion

In this chapter I have shown that in the developing *Drosophila* embryo, NERD is a nervous system-specific gene product.

As reviewed in Chapter I, it has been widely proposed that in vertebrates GAP-43 expression plays a key role in neurite outgrowth and synaptic physiology. For the most part, the suggested role in outgrowth has been based on the observation that GAP-43's appearance in many systems coincides with initial phases of axonal extention, both during ontogeny and during regeneration (Doster et al., 1991; Fitzgerald et al., 1991; Meiri et al., 1991; Sommervaille et al., 1991; Lin et al., 1992). At least one prior study has argued against an indispensable function for GAP-43 in neurite outgrowth. Cultured PC12 cells deficient for GAP-43 are still able to extend neurites in response to nerve growth factor (NGF) (Baetge, et al., 1991). It has also been noted (Meiri, et al., 1991) that only non-phosphorylated forms of GAP-43 are detected in the early phase of neurite development, and that the phosphorylated form is seen much later, when growth cones first enter the field of their synaptic targets. If phosphorylation of GAP-43 was a prerequisite for the activation of a neuronal function, this could therefore occur only later, perhaps during synapse formation.

The localization of *nerd* described in this chapter indicates that expression occurs late in embryonic development. This temporal pattern is not consistent with a role for the putative gene products in the initiation of axonal outgrowth, since at the time of peak mRNA expression (16 hours AEL), neurite outgrowth is largely complete (Campos-Ortega and Hartenstein, 1985). If NERD has a function in the *Drosophila* embryo that is truly homologous to that of the vertebrate proteins, then a role in the establishment or maintenance of synaptic contacts is more plausible for these proteins. The *Drosophila* homologs of the synaptic vesicle proteins rab3 and synaptotagmin (DiAntonio et al., 1993a),

which are essential for synaptic transmission, are expressed in a similar developmental time-frame as the NERDs, reinforcing the idea that NERD (and by analogy GAP-43) may have a primary synaptic role.

How can the very early expression of GAP-43 during mammalian neuronal ontogeny be explained under the assumption that NERD and GAP-43 are performing the same role? One possibility is that although GAP-43 is expressed coincidentally with the first stages of neurite outgrowth, it is "stored" in growth cones until it is required for synaptogenesis. This would obviate a genetic or epigenetic mechanism to trigger production and transport of proteins needed to establish the contact. If the target field and the cell body projecting the axon were far from each other, the lengthy delay required for synthesis and transport might interfere with the timing of synaptogenesis.

Another possible explanation for why GAP-43 is expressed during earlier phases of axon development is that additional signal tranducing events (which may require a GAP-43-like protein) are taking place in the more complex milieu of vertebrate systems than in the highly stereotyped invertebrate system. Vertebrate neuronal development is marked by periods of outgrowth refinement, where a growth cone will extend in one direction, encounter some barrier and reverse it course to extend in a totally new direction (Fujisawa et al., 1982). This re-routing "decision" may involve the utilization of GAP-43.

The placement of NERD within the nervous system strengthens the argument that GAP-43 and NERD may have related, if not homologous, functions.

Chapter IV: Biochemistry of NERD

Introduction

In the previous two chapters I have identified NERD as a nervous system-specific *Drosophila* gene product with homology to the vertebrate protein GAP-43. Though the presence of three GAP43 modules in NERD-L highlights the importance of this 20 amino acid sequence, it is still necessary to demonstrate **functional** similarity between NERD and GAP-43 in order to satisfy the "homolog" argument.

As reviewed in Chapter I, protein kinase C (PKC) is thought to be intricately involved in many phases of neuronal behavior, including neurite outgrowth, neurotransmitter release and synaptic plasticity. Mice mutant for the γ-isoform of PKC (a predominantly postsynaptic form of the kinase) have a diminished capacity to generate LTP in the hippocampus and, perhaps consequently, disrupted spatial and contextual learning abilities (Abeliovich et al., 1993a; Abeliovich et al., 1993b). The key substrates of PKC mediating these phenomenon are not known. At present, GAP-43, neurogranin and MARCKS are the only PKC substrates (Robinson, 1991) whose phosphorylation has been linked to some or all of the PKC-related functions. In particular, GAP-43 seems to be associated with many of the activities that require PKC. In this chapter, the phosphorylation of NERD by PKC is examined.

The accumulation of GAP-43 in the growth cones of developing axons has been shown to require palmitoylation of two N-terminal cysteine residues, as outlined in Chapter I. It is not known if this modification functions solely in protein localization or if an additional function requires this fatty acid linkage. Since palmitoylation is reversible, the possibility remains that the dynamic regulation of lipid addition may be involved with GAP-43 activity. In fact, it has recently been shown that the interaction of GAP-43 with G_0 is inhibited *in vitro*

by palmitoylation (Sudo et al., 1992). Another conspicuous member of synaptosomal membranes is the MARCKS (which stands for Myristoylated Alanine-Rich C-Kinase Substrate) protein (Wang et al., 1989). This protein is acylated, although the attachment is to a myristoyl, not a palmitoyl, moiety on the N-terminal glycine residue (Aderem et al., 1988). It is not known whether this addition is required for targetting MARCKS to the synapse, but myristoylation is required for membrane association (Thelen et al., 1991). I demonstrate that NERD-L, but not NERD-S, is myristoylated *in vitro*.

An interesting property of GAP-43, neurogranin and MARCKS, is an unusual retarded electrophoretic mobility on SDS-PAGE gels. This property is also found in both NERD proteins. The reason for this has been attributed to the unusual amino acid composition (high alanine and charged/polar amino acid content) which decreases SDS-binding, and hence migration in SDS-based gels.

Calmodulin (CaM) and GAP-43 have been known to interact *in vitro* for more than a decade (Andreasen et al., 1983). The CaM-binding domain of GAP-43 coincides with the GAP43 module, and therefore NERD should possess the ability to interact with CaM. However, using *in vitro* translated proteins, I was unable to demonstrate an attachment of NERD proteins to a CaM-sepharose affinity matrix under conditions (Chapman et al., 1991) that did allow GAP-43 to bind (data not shown). Nevertheless, using a yeast interaction trap system, I have identified CaM as the major, if not exclusive, NERD-interacting protein in *Drosophila*.

These four properties - PKC phosphorylation, fatty-acid linkage, CaM-binding, and retarded migration through SDS-gel - coupled with the localization and primary sequence homology are used to argue that NERD-L may represent a homolog of GAP-43 and NERD-S a neurogranin homolog.

Results

PKC phosphorylates NERD on the seryl reisdue of the S module

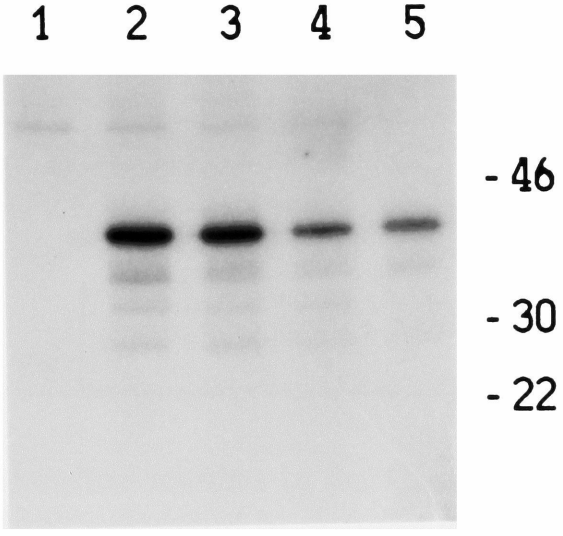
An initial experiment was performed to investigate whether protein kinase C recognized NERD as a substrate. A bacterial fusion protein containing glutathione-S-transferase (GST) and the entire coding region of NERD-S(GST-NERD-S) was combined with purified PKC *in vitro*. As seen in Figure IV.1, GST is not a substrate for the enzyme (lane 1), but GST-NERD-S is rapidly phosphorylated (lane 2). Increasing concentrations of a peptide modeled on the NERD S-module inhibit this process. The experiment demonstrates that NERD is a PKC substrate and the GAP43 module peptide is a PKC inhibitor (*i.e.* alternate substrate) *in vitro*.

The peptide inhibitor (KAATKIQASFRGHKTRK) was also phosphorylated rapidly on serine in the absence of GST-NERDS. In Figure IV.2, the S-module peptide and a mutant peptide with a valine substitution at serine (KAATKIQAVFRGHKTRK) were separately incubated with ^{32}P-γATP and purified PKC. Equal quantities of the phosphopeptides were spotted to P81 cellulose paper and the radioactivity was counted. Approximately ninety percent of the incorporated phosphate was attached to serine. The remaining ten percent probably represented phosphorylation of the C-terminal threonine, which conforms to the major PKC recognition consensus K/R-X-S/T-X-K/R (Kennelly and Krebs, 1991), although the N-terminal threonine also is surrounded by a weak consensus.

In order to determine if the serine of the S-module and perhaps the threonine of the T-module were phosphorylated in the intact protein, GST-NER-L fusions were generated with point mutations in the modules. In one fusion,

Figure IV.1: Recombinant NERD fusion protein is phosphorylated *in vitro* by protein kinase C

Five μg of recombinant glutathione-S-transferase (GST) (lane 1) or GST-NERD-S (lane 2) fusion protein, was incubated for 5 minutes at 30°C with 30ng of purified rat PKC (Promega) in a 50μl reaction containing 20mM HEPES (pH 7.4), 0.4mM EDTA, 0.4mM EGTA, 1.7mM$CaCl_2$, 1mM DTT, 10mM $MgCl_2$, 5μg phosphatidylserine, 0.5μg diolein, 250mM ATP, and 1μl ^{32}P-γATP (3000Ci/mmol). Lanes 3-5 contain 0.1μg, 0.5μg and 4μg, respectively, of the synthetic peptide NH_2-KAATKIQASFRGHKTRK-COOH. This peptide corresponds to the NERD-S module. The reactions were stopped with SDS-loading buffer, boiled, and 5μl was electrophoresed on a 12% SDS-PAGE gel, which was then dried and exposed to X-ray film at -70°C for 4 hours. Lane 1 shows that GST is not a substrate for PKC. The GAP43 module peptide can inhibit the phosphorylation of the NERD fusion.


1
2
3
4
5
- 46
- 30
- 22

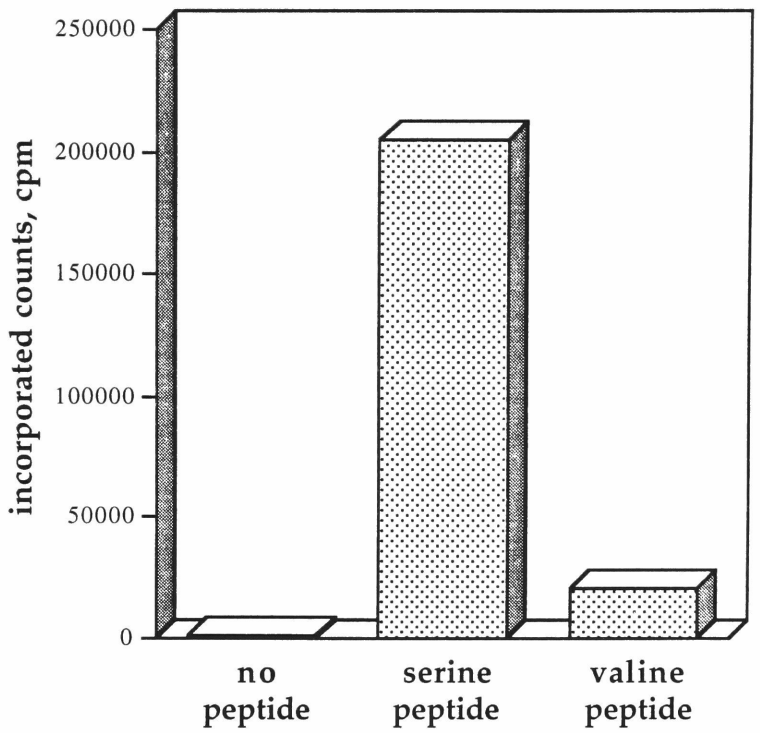


Figure IV.2: The serine residue in the GAP43 module peptide is phosphorylated by protein kinase C

A peptide based on the consensus GAP43 module sequence with either a serine or a valine at the predicted phosphorylation site was synthesized. Eight hundred nanograms of peptide or buffer was added to thirty nanograms of purified protein kinase C (Promega) with the same reaction conditions as in Figure IV.1. After a five minute incubation at 30°C, the reaction was stopped by heating to 70°C for 10 minutes and then spotted to Whatman P81 phosphocellulose paper. After repeated washing in 75 mM phosphoric acid, the paper was dried and counted in scintillation fluid. Each experiment was done in duplicate and averaged.

the S-module serine was converted to alanine (GST-NERD-L:S->A), in another fusion the T-module threonine was converted to a valine (GST-NERD-L:T->V) and in one fusion both mutations were combined in a single protein (GST-NERD-L:S->A/T->V). The fusion proteins were phosphorylated with PKC, fractionated on SDS-PAGE gels, visualized by autoradiography and gel slices corresponding to labeled protein were cut from the gel. Wild-type fusion protein was subjected to one-dimentional phosphoamino acid analysis (see Figure IV.3). Surprisingly, it was evident that the majority of incorporated phosphates were attached to threonine, at a rate of approximately 10:1 threonine:serine as assessed by scanning densitometry (data not shown). A two-dimensional (2-D), thermolytic phosphopeptide map was performed in order to directly determine if the S-module serine and the T-module threonine were being phosphorylated. In Figure IV.4A, a thermolytic map of the wild-type fusion shows at least 8 peptides labeled. In the lower panel, a single acidic spot vanishes from the map of the GST-NERD-L:S->A mutant fusion protein, demonstrating unequivocally that the expected serine is phosphorylated by PKC. The remaining basic spots were scraped from the cellulose plate and individually subjected to phosphoamino acid analysis (data not shown). They all contained only phosphothreonine. The phosphoserine spot on the 1-D separation in Figure IV.3 is therefore entirely accounted for by the S-module serine.

A similar experiment with the T->V mutation, however, generated an apparently wild-type map (Figure IV.4C,) To make sure that the many phosphothreonine spots in the thermolytic map were not masking a deleted phosphothreonine in the mutant fusion, an additional proteolytic map was prepared following digestion with trypsin rather than thermolysin. The result of this analysis was identical, namely the T-module threonine is not recognized by PKC as a substrate (data not shown). This result is not surprising, despite the

Figure IV.3: Protein kinase C phosphorylates several sites on the GST-NERD-L fusion protein *in vitro*

Five micrograms of GST-NERD-L fusion protein were phosphorylated with purified PKC as in Figure IV.1. The reaction was stopped with SDS-loading buffer and electrophoresed on a 12% SDS-PAGE gel, dried and mounted for autoradiography. Visualized phosphoprotein was excised, eluted in 50mM NH_4HCO_3, lyophilized and then hydrolyzed in 6N HCl for 2 hours at 110°C. After lyophilization, the phosphoamino acids were suspended in pH 1.9 buffer. The samples were spotted with standard onto TLC plates and electrophoresed in pH 1.9 buffer followed by pH 3.5 buffer. After drying the plates the standards were visualized with 1% ninhydrin spray. The labeled phosphoamino acids were visualized after an appropriate exposure on X-ray film at -70°C.Figure IV.4:

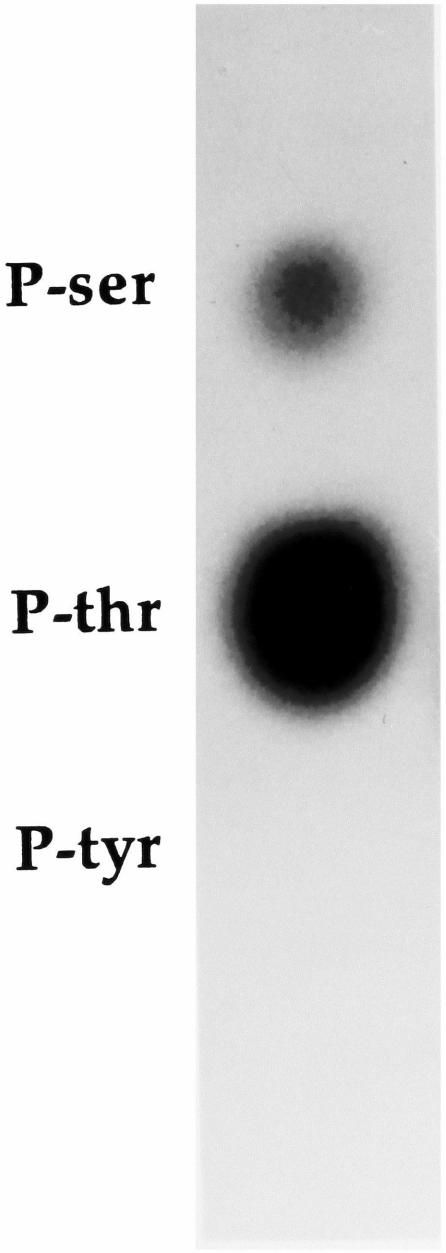
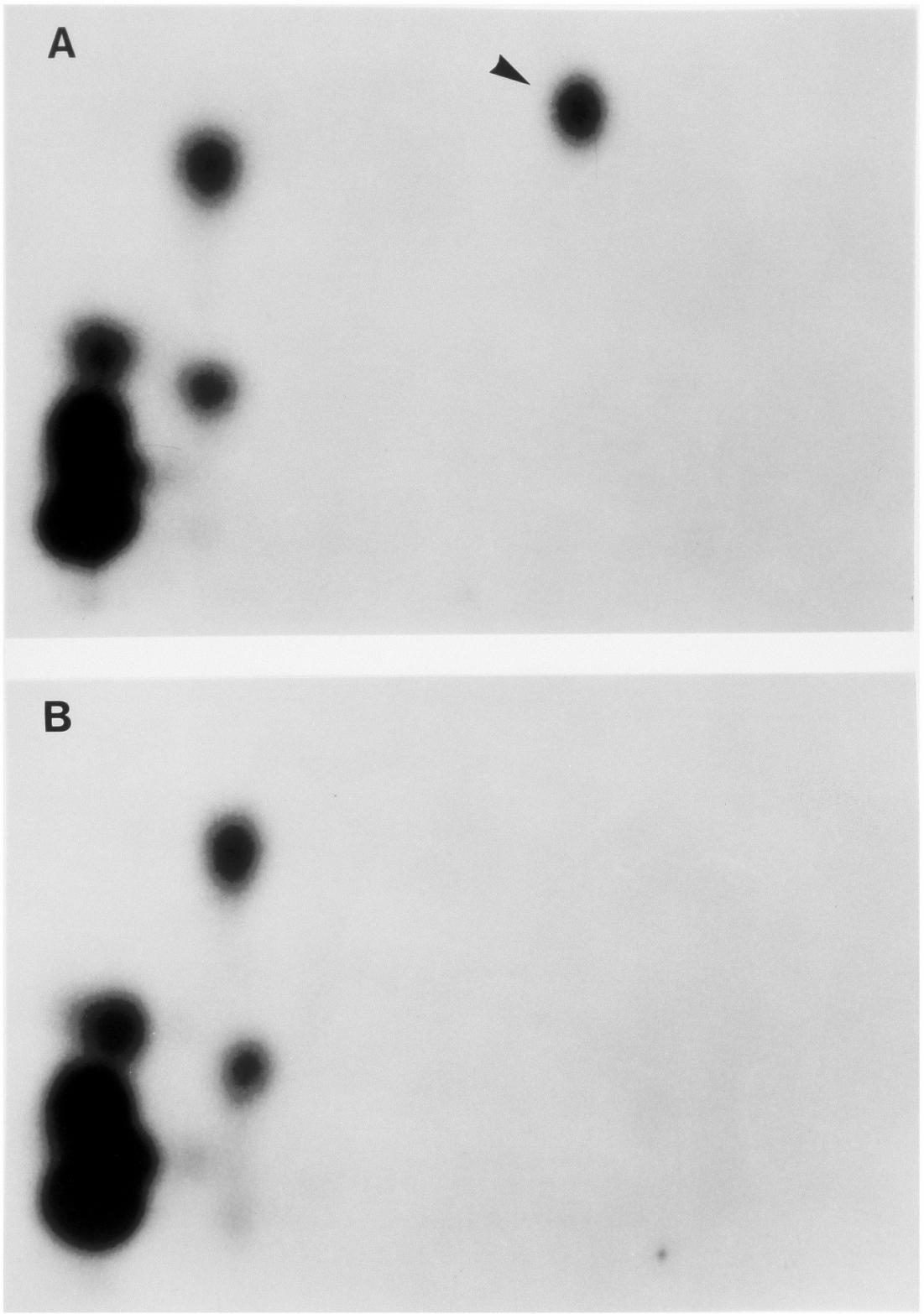
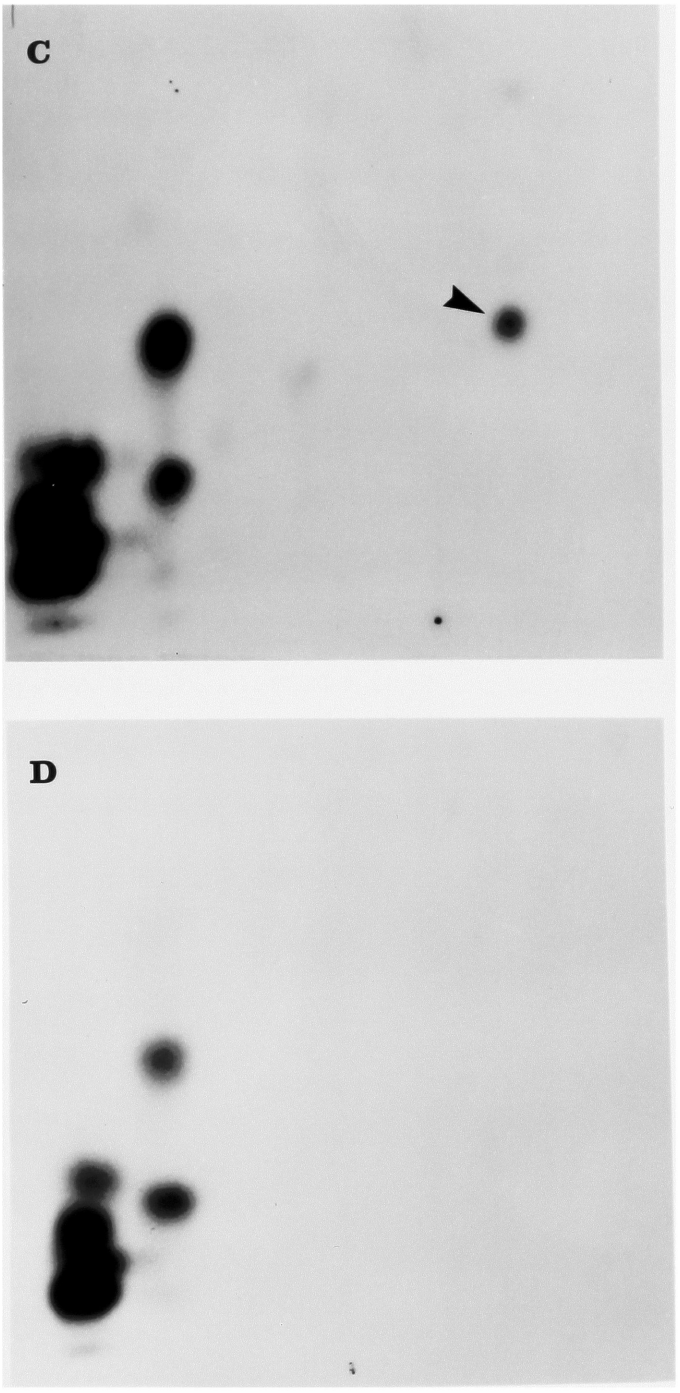

P-ser
P-thr
P-tyr

Figure IV.4: Two-dimensional proteolytic mapping reveals that the S-module serine, and not the T-module threonine, is a protein kinase C substrate *in vitro*

NERD-L was fused to glutathione transferase (GST) and purified by glutathione-affinity chromatography. Five micrograms of the protein were incubated with purified PKC and ^{32}P-γATP for 10 minutes at 30°C as described in Materials and Methods. The phosphorylated fusion protein was purified on a SDS-PAGE gel, excised and digested with thermolysin. The proteolytic fragments were spotted onto cellulose plates analysed in two dimensions. The cathode was on the left in each figure. (A) Wild-type NERD-L thermolytic signature. The serine at position 227 is marked with an arrowhead. In (B) a mutant fusion protein (S227A) was treated as in (A). Note that a single phosphopeptide spot was deleted. Analysis of a T177V, the T-module mutant, gave an identical map as wild-type, as seen in (C). The double mutant, T177V;S227A, was indistinguishable from the S227A single mutant (D), leading to the conclusion that the T-module threonine is not recognized by PKC *in vitro*.


A
B


C
D

fact that PKC can often phosphorylate threonine if it appears in the proper context. Mutation of the GAP-43 serine to threonine abolishes phosphorylation by PKC (Nielander et al., 1990).

At present I am unable to account for the seven phosphothreonine spots on the 2-D maps. The coding region of NERD-L does contain seven threonines that potentially could be phosphorylated, but the sequence surrounding these residues does not match the PKC consensus very well. One possibility is that the fusion of NERD-L to GST reveals cryptic PKC sites in GST. PKC substrates must often be able bind phospholipid prior to PKC phosphorylation (Bazzi and Nelsestuen, 1987). It is known that the GAP43 module can associate with phospholipid membranes through an ionic interaction (Houbre et al., 1991). If GST-NERD-L also associates with phospholipids through the GAP43 module, perhaps it "drags" GST into an environment wherein PKC can phosphorylate one or more of its threonines. Since the goal of the experiments listed above was simply to determine if the serine and/or threonine in the S- and T-modules, respectively, were PKC substrates, I did construct further experiments to reveal the origin of the additional PKC-phosphorylated threonines.

NERD-L is myristoylated in vitro

All vertebrate GAP-43 molecules have retained an amino-terminal sequence that has been shown to harbor a palmitoylation signal. As described in Chapter I, targetting to growth cones requires this lipid modification. The NERD proteins do not contain this consensus. If palmitoylation is specifically necessary for GAP-43 activity, then the NERD proteins cannot fulfill all of the roles of GAP-43. However, it is conceivable that other lipid modification could subserve a membrane targeting role. It was recently pointed out (A. Aderem, personal

communication) that glycine[2] of NERD-L satisfies the minimum consensus for myristoylation (Towler et al., 1988):
(gly)-(no charge,no pro, no bulky hydrophobic res.)-(neutral >basic> acidic, no pro)-(neutral >basic> acidic)-(ser favored)-(no pro, asp/asn allowed)
The NERD-L myristoylation sequence (GCNTSQ) is similar to the G_0 α-subunit sequence (GCTLSA). While the presence of this consensus is suggestive it does not prove that NERD-L is in fact myristoylated.

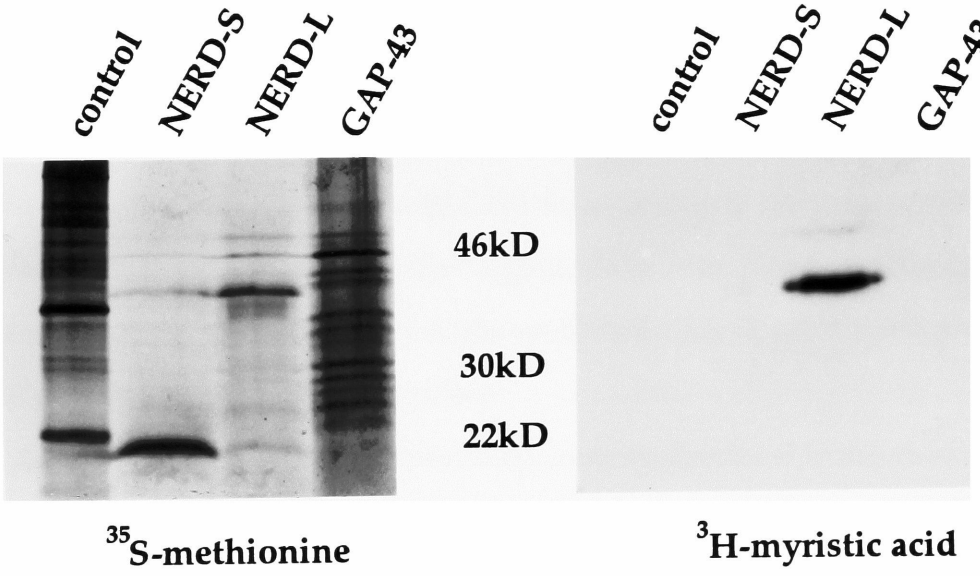
In Figure IV.5, *in vitro* transcribed RNAs for NERD-S, NERD-L and GAP-43 were translated using reticulocyte lysates in the presence of either ^{35}S-methionine or tritiated myristic acid. These lysates contain an active N-myristoyltransferase activity (Li and Aderem, 1992) which can co-translationally attach myristate to substrate proteins. The reaction products were run on SDS-PAGE gels. After the gels were fixed, they were soaked overnight in several changes of 1M hydroxylamine, and then processed for autoradiography. Wild-type NERD-L is covalently associated with the myristic moiety (lane 3). The fact that the fatty acid linkage is resistant to hydroxylamine establishes that the protein is connected to the lipid through an amide bond and not a thioester bond via cysteine which are the route of attachment of palmitoylations and isoprenylations. NERD-S (lane two), which lacks the consensus myristoylation site, fails to bind the fatty acid, as do the control proteins in the first lane and GAP-43 in the fourth lane.

NERD proteins have unusual electrophoretic properties

One biochemical oddity common to both GAP-43 and neurogranin (and the MARCKS protein) is their unusual migration on SDS-PAGE gels. Although GAP-43 runs at an aparent weight of 43kD on 10% gels, the actual molecular weight of the protein is 24kD (Benowitz et al., 1987). Neurogranin runs at 17kD,

Figure IV.5: NERD-L is myristoylated *in vitro*

Rabbit reticulocyte lysates (30µl reactions) were supplemented either 40µCi of ^{35}S-methionine or with 40µCi of ^{3}H-myristic acid and *in vitro* synthesized RNAs were added. The reactions were stopped with SDS-loading buffer, boiled and electrophoresed on 12% SDS-PAGE gels. After fixation, the ^{35}S-methionine gel was soaked in Amplify (Amersham), dried and exposed at -70°C overnight. The tritiated gel was soaked overnight in 1M neutral hydroxylamine after fixation, immersed in Amplify and dried and exposed at -70°C for 3 weeks. The control lane contains brome mosaic virus RNA , as supplied with the lysate. Only NERD-L was found to be covalently attached to a hydroxylamine-resistant fatty acid (see Materials and Methods). Molecular weight markers are expressed in kilodaltons.


control
NERD-S
NERD-L
GAP-43
46kD
30kD
22kD
control
NERD-S
NERD-L
GAP-43
35S-methionine
3H-myristic acid

but is actually 7.8kD (Baudier et al., 1991). This electrophoretic anomaly has been attributed to the inability of the proteins to bind SDS efficiently and the elongated tertiary structure of the molecules (Masure et al., 1986b). The NERD proteins also have this behavior. In Figure IV.5 the *in vitro* translated NERD-L protein migrates at 40kD on 12% gels, whereas the calculated molecular weight of the protein is 25kD. NERD-S, whose molecular weight is 13kD, travels at 20kD.

The three GAP43 modules of NERD independently bind calmodulin

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding peptide involved in a number of important neuronal processes, as reviewed in Chapter I. The vast majority of proteins that interact with CaM do so strictly in the presence of Ca^{2+}. In this way, CaM can transmit the Ca^{2+} signal. Examples of important regulatory enzymes that are activated by Ca^{2+}/CaM are CaM kinase II, calcineurin and Ca^{2+}/CaM-responsive adenylate cyclase.

GAP-43 is one of a few CaM-binding proteins that bind in the *absence* of Ca^{2+}. Indeed there are so few proteins with this property has been exploited as a means of purifying the protein by CaM-sepharose chromotography (Chapman et al., 1991). Attempts to demonstrate a similar ability of NERD proteins to bind CaM-sepharose under conditions that GAP-43 bound (50mM Tris •HCl (pH7.5), 1mM DTT, 5mM EDTA) were unsuccessful (data not shown). One possible explanation for this result was that the fly protein required a *fly* CaM, not a rat CaM, as was used. Despite the fact that the two molecules differ at only three of 148 residues, crystallization studies have shown that there are significant structural differences between mammalian and *Drosophila* CaM proteins (Taylor et al., 1991), with an overall root mean square deviation of 1.22Å for 1,120 atoms.

Changes were noted in the Ca^{2+}-binding domains as well the amino terminus end of CaM.

In order to characterize NERD-binding proteins, and in particular GAP43 module-binding proteins, I implemented a yeast-interaction trap screen based on the method of Fields (Fields, 1993) as modified by Brent (Zervos et al., 1993). The details of this procedure are found in Materials and Methods and the general procedure is schematized in Figure IV.6. A 0-12 hour embryonic library (a gift of R. Finley) was screened with a NERD probe containing amino acids 159 through 240. This region contains the T- and S-modules. A small probe size increases the likelihood that any of the interacting proteins isolated would specifically recognize the GAP43 module.

Four million primary yeast transformants were screened by selection for growth on leucine-minus media. The strength of protein-protein interactions was simultaneously monitored by co-transformation with a lacZ reporter gene and the addition of X-gal to the media. Approximately 400 galactose-inducible blue colonies were isolated. Twelve of these were selected at random and the cDNAs encoding the interacting proteins were rescued and sequenced. All twelve isolates were identical to fly CaM (Yamanaka et al., 1987). The fusion cDNA encoded amino acids 11-148 (data not shown). The remaining 388 yeast colonies were transferred to nitrocellulose filters and hybridized with a labeled fly CaM-coding cDNA probe. All the colonies hybridized strongly, revealing that the interacting proteins recovered from the screen were CaM.

The GAP43 modules of NERD were shown to mediate the *in vivo* CaM interaction in the following series of experiments (Figure IV.7). Based on the observations that phosphorylation of $serine^{41}$ of GAP-43 by PKC abolishes CaM binding (Alexander et al., 1987) and that a site-directed mutation of this serine to aspartate mimics phosphorylation and also disrupts the CaM-GAP-43 interaction

Figure IV.6: Schematic representation of the yeast interaction trap method for identifying interacting proteins

The following method is based on the work of Fields (Fields, 1993) and Brent (Zervos et al., 1993), and can be summarized as follows.

In-frame fusions are created between a DNA-binding protein (LEXA) and a sequence of interest, called the "bait"(NERD). A construct bearing this fusion and an appropriate selectable marker, is introduced into a yeast strain. A second plasmid harboring a LEXA DNA-binding motif upstream of the lacZ gene is co-transformed with the bait. The LEXA protein targets the fusion to the yeast nucleus where it binds the operator sequence upstream of lacZ. However, LEXA and the bait are not capable of stimulating transcription from the lacZ promoter region (This is determined empirically).

A library containing fusions of the GAL4 transactivating domain and random cDNAs is introduced on a third selectable plasmid into the doubly-transformed strain described above. On average, one library plasmid enters a yeast cell. If that plasmid contains a cDNA product (target) that can bind the "bait"(NERD) then a hybrid complex is formed which can summarily activate transcription from the lacZ gene. The result is a blue colony in a sea of white colonies. Proper controls include the determination that the rescued library protein is interacting with the "bait" and not with the LEXA protein, and conversely that the "bait" is specifically interacting with the "target" and not the GAL4 sequence.

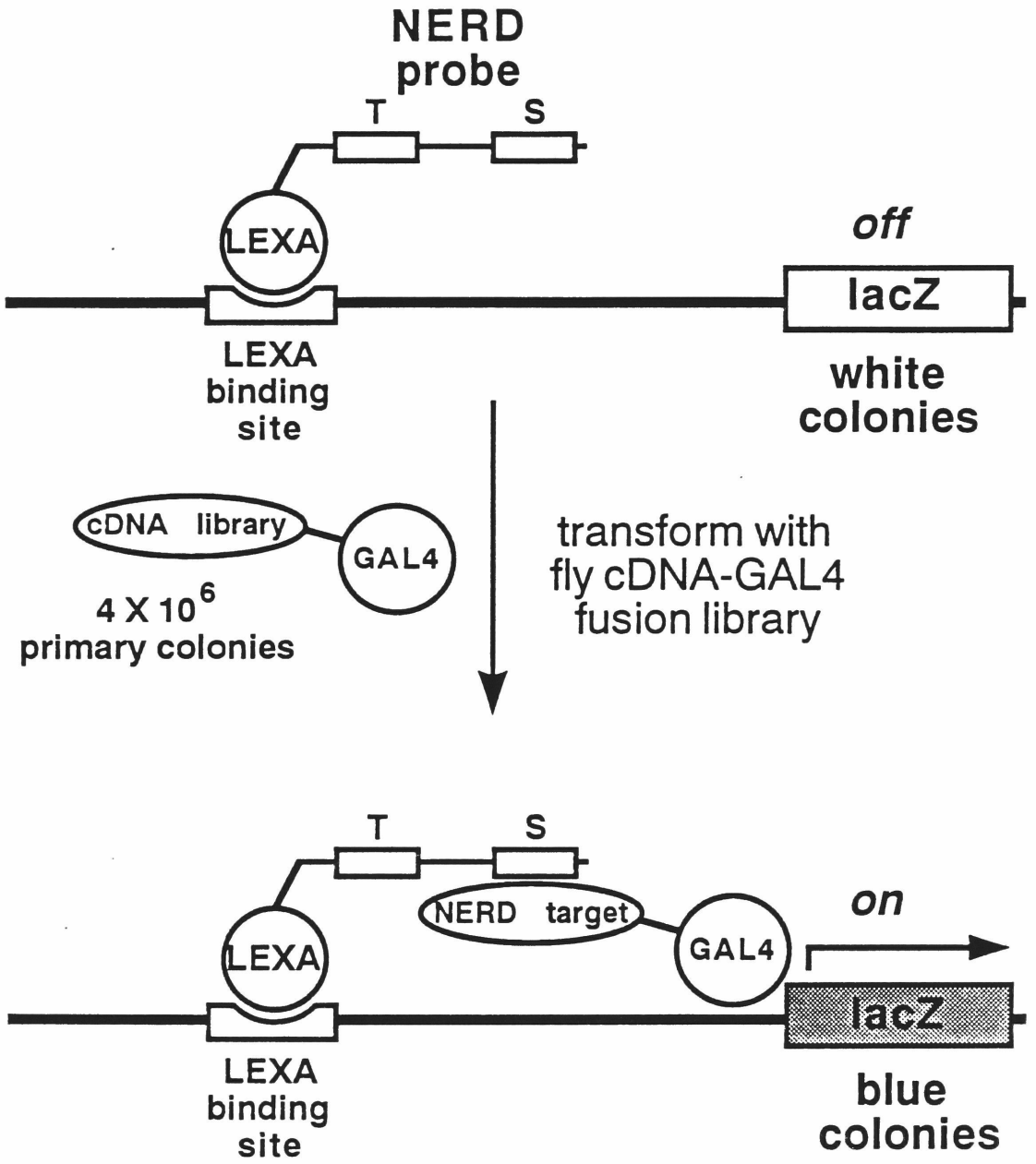
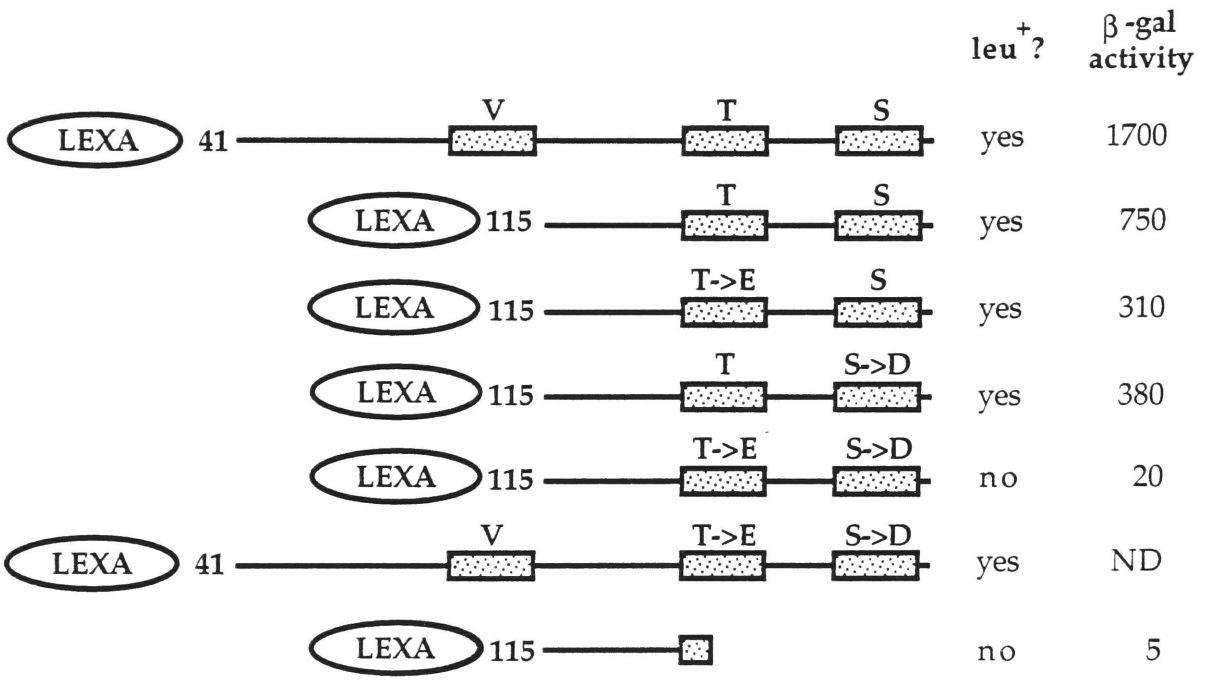

NERD
probe
T
S
LEXA
off
lacZ
LEXA
binding
site
white
colonies
cDNA library
GAL4
4 X 10^6
primary colonies
transform with
fly cDNA-GAL4
fusion library
T
S
NERD target
GAL4
on
LEXA
lacZ
LEXA
binding
site
blue
colonies

Figure IV.7: The three NERD modules bind calmodulin in a pseudophosphorylation-sensitive manner

Various deletions encoding the NERD-L protein were fused in-frame to the LEXA protein in the plasmid pEG202 (see Materials and Methods). These constructs were then transformed into the leu$^-$ yeast strain EGY48. In this strain the LEU2 promoter sequence has been deleted and replaced with tandem LEXA binding sites. EGY48 was also transformed with pLBN6.15 and pSH18-34. pLBN6.15 is an isolate from a 0-12 hour *Drosophila* library (a gift of R. Finley) and is a B42 "acid patch" activation domain-CaM fusion under the GAL1 galactose-inducible promoter. pSH18-34 is a *lacZ* reporter plasmid with eight LEXA binding sites in the promoter region. A protein interaction between a NERD-L construct and the CaM fusion protein results in the transient formation of functional DNA binding(LexA)/trans-activating (B42) protein which can activate the LEU2 gene in EGY48 and the lacZ gene on pSH18-34. LEU2 activity was measured as visible growth on leucine dropout minimal media plates after five days of incubation. ß-galactosidase activity was measured as described in Materials and Methods. ND=not done. Mutant GAP43 modules are denoted T->E for the replacement of thr^{177} with glutamate and S->D for the replacement of ser^{227} with asparate as described in Materials and Methods and discussed in the text.


leu+?
β-gal activity
LEXA
41
V
T
S
yes
1700
LEXA
115
T
S
yes
750
LEXA
115
T->E
S
yes
310
LEXA
115
T
S->D
yes
380
LEXA
115
T->E
S->D
no
20
LEXA
41
V
T->E
S->D
yes
ND
LEXA
115
no
5

in vitro (Chapman et al., 1991), I made analogous mutations in the GAP43 modules of NERD and transformed them into the yeast system. β-galactosidase activity measurements give an estimate of the strength of the protein-protein interaction. Pseudophosphorylation mutations converting the T-module threonine to glutamate or the S-module serine to aspartate reduce the CaM-NERD interaction by approximately 50%. A double-mutant completely abolishes the interaction. Addition of the V-module to the double mutant fusion restores leu^+ activity, though β-galactosidase activity was not measured with this construct.

The intracellular concentration of Ca^{2+} in the yeast cell nucleus, where the trap interaction is occuring, has not been reported. However, the resting cytosolic Ca^{2+} concentration in the yeast cell is approximately 50nM (Halachmi and Eilam, 1993), similar to the resting concentration in vertebrate cells. At this concentration, the majority of CaM is not complexed with Ca^{2+}. It is likely therefore, that the NERD/CaM interaction is occuring in the absence of Ca^{2+}.

These results confirm that CaM-binding detected in this system is achieved through a direct interaction with the GAP43 module sequence of NERD. All three modules are capable of binding CaM (although V-module binding was only inferred) and mutations that introduce a negative charge in place of the canonical serine inhibit CaM binding *in vivo*. This artificial modification can mimic phosphorylation in some cases (Chapman et al., 1991).

Discussion

A number of biochemical properties associated with GAP-43 have now been identified in the NERD proteins. Since PKC phosphorylation and CaM-binding had previously been shown to map to the conserved GAP43 module, it is not unexpected that these properties are resident in the multiple-module NERD

proteins. It is no less surprising that the single module-containing protein neurogranin shares these activities with GAP-43 and the NERDs.

As GAP-43 has been proposed to act in growth cones and in presynaptic membranes, a crucial determinant of its function must be its ability to localize properly. The detailed mechanism of growth cone targeting is unknown, but fatty acylation is necessary (but not sufficient, see (Liu et al., 1991)) for the efficient transfer of GAP-43 to neuronal membranes.

Because the NERDs lack the palmitoylation site conserved between fish and mammals, it was necessary to postulate an alternate mechanism for NERD localization. Such a mechanism need not include an obligatory acylation event, because although lipid modification can assist in membrane attachment, acylation *per se* does not always lead to membrane attachment.

MARCKS is an example of a protein that does use acylation to drive membrane association (Graff et al., 1989). Moreover this protein, like GAP-43, is transported to pre-synaptic membranes. While it has not been established, it is conceivable that, analogous to the palymitoylation of GAP-43, the myristoylation of glycine[2] in MARCKS may assist in this trafficking task. In addition to MARCKS, the membrane-associated tyrosine kinase c-src is myristoylated (Schultz et al., 1985) and transported to the growth cone with kinetics very similar to GAP-43 (Maness et al., 1988). Myristoylation and palmitolyation do not always lead to membrane association since some cytosolic proteins contain these additions (Carr et al., 1982). While it cannot be argued that N-terminal acylation is the *sine quo non* of growth cone targeting in neurons, it is conceivable that certain classes of proteins can utilize this modification to foster proper localization.

If the palmitoylation of GAP-43 and the myristoylation of NERD-L serve a similar or identical function, namely as growth cone/pre-synaptic localization

signals, then it suggests that a primordial "GAP-43"-like molecule, one that existed before invertebrates and vertebrates diverged, contained neither signal, for if a functional signal had existed there would have been a very strong selection pressure to maintain it. Therefore, it is possible that during evolution the vertebrate and invertebrate lineages developed different acylation signals to meet a common need. It will be interesting to see if other invertebrate species, for example *Aplysia* , contain NERD-related homologs, and if these proteins have myristoylation signals, as would be predicted.

GAP-43, neurogranin, MARCKS, MacMARCKS and the NERD proteins share an interesting property that derives not from their primary sequence similarites, but instead from their amino acid composition. In all of the proteins, proline, alanine, lysine, glutamate/glutamine, and aspartate/asparagine make up 70-80% of the amino acids outside of the protein interaction domains (GAP43 modules in GAP-43, neurogranin and the NERDS, and the effector domain of MacMARCKS(42K)/MARCKS (Seykora et al., 1991) (Li and Aderem, 1992)). Since they are very acidic, they bind SDS poorly and this has been offered as a partial explanation for their unusual retardation in SDS-PAGE gels (Benowitz et al., 1987). Typically, the proteins run at 1.5-2.5 times their true weights, depending on the percentage of the gel. Their lack of aromatic residues and bulky hydrophobic residues make the proteins extremely soluble, even in 2.5% perchloric acid. Moreover, where it has been studied, they lack an organized secondary structure and so exist as elongated, random coils in solution (Masure et al., 1986a). These observations give rise to the notion that outside of the effector domains of these molecules, the primary sequence of the proteins is not as important as the the chemical composition of the proteins. This explains the rapid evolutionary changes when comparing mammalian, fish and *Drosophila* GAP-43/NERD molecules. It is unlikely that these regions have a role in

protein:protein interactions or an enzymatic role, but rather may provide a backbone structure for the presentation of their effector (GAP43 module) domains. It would be interesting to see if adding a GAP43 module to a MARCKS backbone would yield a GAP-43-like protein.

What would be the utility of such a structure? The elongated axial structures impede progress through lattice-like supports such as chromatography columns or acrylamide gels. It may not be a coincidence that the growth cone is a similar terrain, brimming with actin filaments and cross-linking proteins. Structurally, the growth cone may resemble a lattice support. The experimentally observed impedance of GAP-43/MARCKS-like proteins may have the *in vivo* role of limiting the diffusion of the proteins. Since GAP-43 and MARCKS, and by assumption NERD, are proposed to be signal transducing molecules, the limited mobility seen in gels may reflect a requirement for the limited mobility of molecules once they become activated *in vivo*. For instance, if phosphorylation state of GAP-43 indicated "local membrane activation", it might be harmful to have such a signal wandering around the cell. If it were trapped by means of its elongated and immobile structure, it could stand as a beacon, interacting locally with the proteins mediating the changes associated with growth cone adhesion or mobility, for instance. The prediction of this model is that simply adding the effector domain of GAP-43/NERD or MARCKS to a conventional globular protein would lead to improper utilization of the effector function. This could explain the apparent divergence of GAP-43 and NERD - what is crucial to the appropriate activity of the effector modules is highly conserved, namely a very hydrophilic, elongated amino acid backbone. From this point of view GAP-43 and NERD, and neurogranin for that matter, are *highly* related.

The work represented in this chapter confirms the notion that GAP-43, working via the GAP43 module, interacts with CaM. Mutations in the module that introduce a negative charge in place of the serine of the S-module or threonine of the T-module interrupt the interaction and thus mimic the phosphorylation of the GAP43 module by PKC. As discussed in Chapter I, a predominant theory of GAP-43 action suggests that GAP-43 serves as a CaM "store." Release of the protein in the presence of calcium or following phosphorylation by PKC would lead to the activation of resident CaM-binding proteins (Liu and Storm, 1990). Two of the most troubling aspects of the theory center on the nature of the GAP-43:CaM interaction itself. First, the dissociation constant for GAP-43 and CaM in low salt buffer (50mM Tris•HCl) is in the micromolar range. Most bonafide CaM-binding proteins have nanomolar affinity for CaM in the presence of high salt buffers. Second, although in low ionic buffer the CaM dissociation constant is lower in the absence of calcium than in the presence of calcium (0.2μM v. 1.0μM) the addition of 100mM KCl leads to an increase in both dissociation constants to 3μM (Alexander et al., 1987). This means that in physiological salt, for example, there would be no calcium-mediated regulation of CaM:GAP-43 binding. It is hard to reconcile these facts with the proposed CaM-sequestration model for GAP-43 function.

Is there a significance to the observation that GAP-43/NERD proteins bind CaM weakly? The fact that calcium "selectively" disrupts the interaction *in vitro* is likely to be irrelevant *in vivo*, for the reason given above. The interesting correlation that PKC-mediated phosphorylation also disrupts CaM binding and the *in vivo* implications of that are predicated on the existence of a CaM-GAP-43 interaction *in vivo*. Spencer and Willard (Spencer and Willard, 1992) have hypothesized that GAP-43 could accelerate the transfer of CaM down the axon to reach the growth cone by "piggy-backing" on a rapidly transported protein such

as GAP-43. There is no direct data supporting this view. In fact, CaM is transported by a slow axonal targetting mechanism (Brady et al., 1981). The "piggy-backing" theory, like the CaM-sequestration theory, would seem to hinge on the existence of a stable GAP-43:CaM interaction *in vivo*. While it is experimentally possible to establish conditons that permit CaM-GAP-43 or CaM-NERD associations, the interaction is entirely ionic and dissipated in physiological salt. Although my work demonstrates an interaction between NERD and CaM, I am not able to quantify the strength of this interaction *in vivo*.

In summary, this chapter represents a comparative biochemical analysis of NERD with the known activities of GAP-43. Similarities between the two proteins include *in vitro* CaM-binding, PKC phosphorylation at identical sites, fatty acylation of N-terminal residues and altered biophysical characteristics due to atypical amino composition. In addition to a primary sequence similarity (GAP43 module) and the predominently neuron-specific expression of the proteins, the case can be made that if the proteins are not homologs, they are are at the very least functionally related.

In mammals, two proteins have emerged that share the GAP43 module, namely GAP-43 and neurogranin. The two proteins resemble each other in many ways, including a correlation of LTP formation and PKC-mediated phosphorylation (Gianotti et al., 1992; Klann et al., 1992), but one major difference is their subcellular localization. GAP-43 is primarily presynaptic and neurogranin is postsynaptic (cell bodies and dendrites) (Represa et al., 1990). It has been suggested therefore, that both proteins may perform the same function in different locations within the neuron (Baudier et al., 1991). One distinguishing biochemical feature is that GAP-43 is acylated and neurogranin is apparently not acylated. Could it be a coincidence that in *Drosophila*, a single gene, *nerd*, encodes two overlapping proteins, one acylated and one not, with functional similarity to

GAP-43/neurogranin. In the future antibodies to NERD-L will allow us to distinguish the subcellular localizations of NERD-L and NERD-S, but it is tempting to speculate that NERD-L will be presynaptic (GAP-43-like) and NERD-S will be postsynaptic (neurogranin-like) based on the acylation modification.

Chapter V: Targets for phosphoNERD

Introduction

In the previous chapter, I presented a collection of biochemical data that strengthens the argument that GAP-43 and the NERDs may have similar functions. As reviewed in Chapter I, GAP-43 has been associated with many neuronal processes, but its function is unknown. The original theory that GAP-43 acts as a CaM store and releases it upon calcium influx (Andreasen et al., 1983) has insinuated itself into the neurobiology field and taken the status of fact. Since it is universally held that GAP-43, PKC, CaM and calcium all have important roles in neurite outgrowth, neurotransmitter release and synaptic plasticity, the theory nicely ties together many of the major molecular players in these intensively studied phenomena. It is impossible to give support to this theory because it most important element, namely that GAP-43 can form a stable interaction with CaM, is most likely incorrect.

The IQ motif, which is repeated three times in NERD-L and twice in NERD-S, is a protein:protein interaction domain (Cheney and Mooseker, 1992). Since I was unable to isolate proteins that bound to NERD, other than CaM, there are two possibilities to examine. One possibility is that the library I screened was incomplete. This is likely for two reasons. One reason is that it was synthesized using an oligo-dT linker to prime the mRNA, and therefore only contains the 3' ends of cDNAs. For this reason a large protein that interacts with NERD through a N-terminal peptide sequence would not be represented in this interaction library. A second reason would be that the developmental staging of the cDNAs used in the library did not precisely overlap with maximal NERD expression. The co-expression of interacting proteins might theoretically be regulated temporally and spatially.

A second possibility is that there *are* no proteins that interact with the wild-type GAP43 module. The function of GAP-43 is might be fused to a protein interaction of the phosphorylated form of the molecule. There is a battery of evidence supporting this notion. In an elegant study using a monoclonal antibody that specifically recognized the GAP-43 phosphoprotein, Meiri *et al* (Meiri et al., 1991) showed a temporal and spatial restriction of phosphoGAP-43. Specifically, the phosphorylated protein appears in developing growth cones long after the initiation of neurite outgrowth. Prior to this late phase of axonal evolution, there is a large amount of GAP-43 immunoreactivity, but the vast majority of this pool is not phosphorylated. Baetge *et al.* have isolated a PC12 cell line deficient in GAP-43 but competent to make neurites. The neuritic growth cones that develop however have a low substrate adhesivity. While transfection of wild-type GAP-43 rescues this adhesion defect, transfection of GAP-43 with the PKC-site serine mutated to alanine does not, implying the capacity to be phosphorylated is tied to the missing protein's function. In the adult animal, many brain areas retain moderate levels of GAP-43, but phosphoGAP-43 is detected only in regions thought to undergo activity-dependent synaptic alterations, such as the hippocampus (Neve et al., 1988). This implies either that unphosphorylated GAP-43 has a separate phosphorylation-independent function or that it is "waiting" to be activated by phosphorylation.

In this chapter, I introduce a protein called dART1 that specifically interacts with a pseudophosphorylated form of NERD. A function for dART1 is proposed.

Results

The interaction trap system was employed successfully to isolate CaM as a NERD-binding protein (see Chapter IV). Acidic mutations in place of the canonical serine of the GAP43 module decrease the affinity of NERD for CaM as effectively as phosphorylation of serine by PKC (Alexander et al., 1987) (Chapman et al., 1991). Figure IV.7 illustrates this phenomenon. The LexA fusion containing the double pseudophosphorylation mutant is incapable of associating with CaM. Because the yeast system is extremely sensitive, the lack of β-galactosidase activity associated with this construct suggests that even transient interactions with CaM are not taking place. Operationally, under these conditions pseudophosphorylation can mimic phosphorylation , as it does in other systems (Fong et al., 1989). It was thus conceivable that the positive binding of pseudophosphoNERD to an acceptor protein might be detected in the yeast system. The double mutant was used as a probe instead of the single mutant in order to eliminate CaM "contaminants." Neither module of this probe could bind CaM. The same library used to identify CaM as a NERD target was screened at a similar density. Instead of the hundreds of CaM clones that were isolated with the wildtype NERD probe, only 20 colonies survived on leucine-minus media. Eighteen of these were found to be galactose-dependent and the library cDNAs for each of them was cloned. All of the clones were identical. For reasons I shall give later, this protein is called dART1.

In order to test the specificity of the dART interaction with pseudophosphoNERD, a representative clone was tranformed with a wildtype NERD fusion, a single thre->glu pseudophosphoNERD fusion, and as confirmation of the original result, the double mutant. These results are represented in Figure V.1. The wild-type modules are inactive, demonstrating

that the pseudophospho residue of the GAP43 module is crucial in dART1 binding.

After several attempts, direct *in vitro* confirmation of a phosphoNERD/dART interaction has not been obtained. In one experiment purified PKC was used to phosphorylate, and simultaneously label, a GST-NERD fusion. Then a maltose-binding protein (MBP, New England Biolabs)-dART1 fusion was added, and the reaction was precipitated with amylose resin, which specifically bound to the MBP-dART1 protein. MBP-dART1 did not precipitate GST-phosphoNERD (data not shown). Since this experiment was conducted with recombinant *E.coli* fusion proteins, a plausible excuse for this result could be that bacteria do not produce active proteins.

One specul;ative explanation which should be addressed in the future is the following. Two-dimensional phosphotryptic mapping of *in vitro* phosphorylated NERD revealed a number of unpredicted and unidentified phosphothreonines. One potential PKC-site is on thr^{233} of NERD-L (see Figure II.5). This residue is in a good context for PKC phosphorylation (KTRK) and lies *within* the S-module. Although this residue is not a highly conserved member of the GAP-43/neurogranin/NERD family- it is absent in neurogranin - it does sit next to two perfectly conserved basic residues, arg^{234} and lys^{235}. Whether thr^{233} is a target of PKC *in vivo* is unknown. However, if this threonine is phosphorylated *in vitro* it is possible that it would perturb the function of the module in dART1 binding. There is some evidence that this threonine is in fact an *in vitro* target for PKC. In Figure IV.2, replacement of serine with valine in the GAP43 module peptide blocked phosphorylation by only 90%, implying that another residue, most probably the threonine in question, was a PKC target. GAP-43 has a similarly placed threonine but it is flanked by an isoleucine (ITRK)

not a lysine. It is therefore a less likely PKC target since it is surrounded by fewer basic residues.

Other explanations for a failure in detecting an *in vitro* association between dART1 and NERD are 1) the interaction requires a cofactor, *i.e.* calcium or lipid and 2) the interaction is an artifact of the yeast system. Ultimately, the dART1:NERD interaction **must** be properly measured *in vitro*.

The conceptual translation of the sequence of dART1 was compared to the GenBank data library to find related proteins. Two proteins surfaced from this analysis, FUR1 and URK1, the former being more highly related to dART1 than the later (Figure V.2). FUR1 is the structural gene for yeast uracil phosphoribosyltranferase (UPRTase) (Kern et al., 1990), an enzyme that catalyzes the addition of uracil and phosphoribosyl-pyrophosphate (PRPP) to produce uridine monophosphate (UMP). URK1 is the structural gene for yeast uridine kinase, which catalyzes the reaction uridine and ATP to UMP. Although there is no explicit report in the literature, the uridine kinase activity of URK1 maps to the the N-terminal half of the protein, while the homology to dART1 and FUR1 maps to the C-terminal region (see Figure V.3), suggesting that URK1 encodes a bifunctional enzyme.

Figure V.4 is a compilation of known UPRTases from very different species: *Streptococcus salivarius*(gram-positive bacteria), *Escherichia coli* (gram-negative bacteria), and *Mycoplasma hominis* (mycoplasma) in addition to *S.*

```
dART1:    28 EEILAEYGSNLKLLECNSQVAELLTILRDKNTTRSDFKFYADRLIRLVIEESLNQLPYTH 87
             +++ +E   N+ LL   +Q+  L TI+R+KNTTR DF FY+DR+IRL++EE LN LP
FUR1:     34 QKMSSEPFKNVYLLPQTNQLLGLYTIIRNKNTTRPDFIFYSDRIIRLLVEEGLNHLPVQK 93

dART1:    88 CDVETPTGAIYEGLKYRSGNCGVSIIRSGEAMEQGLRDCCRSIRIGKILVESDANTHEAR 147
               VET T   +EG+ +    CGVSI+R+GE+MEQGLRDCCRS+RIGKIL++ D  T   +
FUR1:     94 QIVETDTNENFEGVSFMGKICGVSIVRAGESMEQGLRDCCRSVRIGKILIQRDEETALPK 153

dART1:   148 VVYARFPDDIGSRQVLLMYPIMSTGNTVLQAVNVLREHGVPESCIILSNLFCTPIAARTV 207
             + Y + P+DI  R V L+ P+++TG + + A  VL + GV    I   NL C+
FUR1:    154 LFYEKLPEDISERYVFLLDPMLATGGSAIMATEVLIKRGVKPERIYFLNLICSKEGIEKY 213

dART1:   208 VNAFPKLKILTSEL 221
               AFP+++I+T  L
FUR1:    214 HAAFPEVRIVTGAL 227
```

Figure V.2: dART1 is related to FUR1, an enzyme from *Saccharomyces* involved in pyrimidine metabolism

A conceptual translation of the dART protein isolated in the interaction trap screen was compared to GENBANK data files using the BLASTP program. FUR1 was the highest scoring entry, with $P(1)=10^{-55}$ (P is the probability of a chance homology). Residues which are identical between the proteins are listed in bold on the line separating dART1 and FUR1. A '+' indicates a conservative amino acid change.

```
URK1        MSHRIAPSKERSSSFISILDDETRDTLKANAVMDGEVDVKKTKGKSSRYI
                                                            ..
URKe                                                        MT

                    ATP-binding
URK1        PPWTTPYIIGIGGASGSGKTSVAAKIVSSINVPWTVLISLDNFYNPLGPE
            .......IIGI.GAS.SGK...A.....................
URKe        DQSHQCVIIGIAGASASGKSLIASTLYRELREQVGDEHIGVIPEDCYYKD

URK1        DRARAFKNEY--DFDEPNAINLDLAYKCILNLKEGKRTNIPVYSFVHHNR
            .............D.P.A....L.......LK.G.....PVYS.V.H.R
URKe        QSHLSMEERVKTNYDHPSAMDHSLLLEHLQALKRGSAIDLPVYSYVEHTR

URK1        VPDKNIVIYGASVVVIEGIYALYDRRLLDLMDLKIYVDADLDVCLARRLS
            ............V...EGI..L.D.RL.D.....I.VD..LD.CL.RR..
URKe        MK-ETVTVEPKKVIILEGILLLTDARLRDELNFSIFVDTPLDICLMRRIK

URK1        RDIVSRGRDLDGCIQQWEKFVKPNAVKFVKPTMKNADAIIPSMSDNATAV
            RD...RGR..D....Q..K.V.P....F..P....AD.I.P....N..A.
URKe        RDVNERGRSMDSVMAQYQKTVRPMFLQFIEPSKQYADIIVPRGGKNRIAI

URK1        NLIINHIKSKLELKSNEHLRELIKLGSSPSQDVLNRNIIHELPPTNQVLS
            ......I....E
URKe        DILKAKISQFFE

URK1        LHTMLLNKNLNCADFVFYFDRLA***TILLSWALDDIPVAHTNIITPGEHTME***

URK1        ***NVIACQFDQVTAVNIIRSGDCFMKSLRKTIPNITIGKLLIQSDSQTGEPQ***

URK1        ***LHCEFLPPNIEKFGKVFLMEGQIISGAAMIMAIQVLLDHGIDLEKISVVV***

URK1        ***YLATEVGIRRILNAFDNKVNIFAG***MIISREKLQNHQYKWALTRFFDSKYF

URK1        GCD
```

Figure V.3: URK1 encodes a bifunctional enzyme with homology to uridine kinase and UPRTase

Yeast uridine kinase (URK1) was also identified as having a significant homology to dART1. This homology overlapped the FUR1 homology and was confined to the C-terminal half of the protein (bold and italic lettering). The first half of the URK1 protein is homologous to the *E.coli* uridine kinase (URKe). The putative ATP-binding site for the kinase is indicated. The N-terminal half of URK1 is not a UPRTase, as is FUR1, since there is no detectable UPRTase activity in *fur1* null mutations (Kern et al., 1990).

```
URKc     TMLLNKNLNCADFVFYFDRLATILLSWALDDIPVAHTNIITPGEHTMENVIACQFDQV
STR      SILRREDTSTKDFRELVNEIAMLMGYEVSRDLPLEEVEIQTPITKTVQKQLSGKKLA-
UPP      GLMREQDISTKRFRELASEVGSLLTYEATADLETEKVTIEGWNGPVEIDQIKGKKIT-
MYCH     TTMRSKETSYKDFRDNLNEIASLMVYETLRDYQTKKISITTPMNVKYEGETLDREIV-
FUR1     TIIRNKNTTRPDFIFYSDRIIRLLVEEGLNHLPVQKQIVETDTNENFEGVSFMGKIC-
dART1    TILRDKNTTRSDFKFYADRLIRLVIEESLNQLPYTHCDVETPTGAIYEGLKYRSGNC-
CON      ...R...TS.KDFR....EI..L..YE...DL...K..I.T.............KI

URKc    TAVNIIRSGDCFMKSLRKTIPNITIGKLLIQSDSQTGEPQLHCEFLPPNIEKFGKVFLM
STR     -IVPILRAGIGMVDGFLSLVPAAKVGHIGMYRDEETLEPVEYLVKLPEDIDQRQ-IFVV
UPP     -VVPILRAGLGMMDGVLENVPSARISVVGMYRNEETLEPVPYFQKLVSNIDERM-ALIV
MCYH    -IIPILRAGLGMLNGIMNLVPQARVGHIGMYRNEETNEVVEYFFKIPEVPHDSY-IIIV
FUR1    -GVSIVRAGESMEQGLRDCCRSVRIGKILIQRDEETALPKLFYEKLPEDISERY-VFLL
dART1   -GVSIIRSGEAMEQGLRDCCRSIRIGKILVESDANTHEARVVYARFPDDIGSRQ-VLLM
CON     -.VPILRAG.GM..G....VP.AR.G.IGMYR.EET.EPV.Y..KLPE.I..R.-...V

URKc    EGQIISGAAMIMAIQVLLDHGIDLEKISVVVYLATEVGIRRILNAFDNKVNIFAGMIIS
STR     DPMLATGGSAILAVDSLKKRGAANIKF--VCLVAAPEGVKKLQDAHPDIDIYTASLDEK
UPP     DPMLATGGSVIATIDLLKKAGCSSIKV--LVLVAAPEGIAALEKAHPDVELYTASIDQG
MYCH    DPMLATGGSACDAIAKLDKLGFNNIKL--VCLVGVQQGIDKVTKQFPNVDIYLASKDEK
FUR1    DPMLATGGSAIMATEVLIKRGVKPERIYFLNLICSKEGIEKYHAAFPEVRIVTGALDRG
dART1   YPIMSTGNTVLQAVNVLREHGVPESCIILSNLFCTPIAARTVVNAFPKLKILTSELHPV
CON     DPMLATGGSAI.A...L.K.G...IK.....LV...EGI.K...A.P.V.IYTAS.D..
     *PRPP-binding*

URKc    REKLQNHQYKWALTRFFDSKYFGCD
STR     LNENGYIVPGLGDAGDRLFGTK
UPP     LNEHGYIIPGLGDAGDKIF
MYCH    LNEHNYILPGLGDAGDRIFGTKIK
FUR1    LDENKYLVPGLGDFGDRYY
dART1   APNHFGQNTSVQTRYLGLSRRLDD
CON     LNE..YI.PGLGDAGDR.F
          *uracil-binding*
```

Figure V.4: Consensus between known UPRTases reveals that dART1 has lost two conserved functional domains

Four sequences from distantly related species and having known biochemical UPRTase activity were retrieved from GENBANK and are abbreviated as follows: STR, *Streptococcus salivarius* UPRTase; UPP, *Escherichia coli* UPRTase; MYCH, *Mycoplasma hominis* UPRTase; FUR1, *Saccharomyces cerevisiae* UPRTase. URKc is the C-terminal region of URK1 uridine kinase (see text). A 100 amino acid consensus sequence (**CON**) was defined wherever at least three of the four confirmed UPRTases had an identical residue. The PRPP (phosphoribosyl-pyrophosphate)-binding site and the putative uracil-binding site are indicated.

cerevisae (fungi). The C-terminal half of URK1 that contains dART1 homology is also included. A consensus was determined using the criterion that at least three of the four known UPRTases must contain an identical residue in a given position. Conservative changes where not included in the analysis in order to maximize stringency. By comparing dART1 to the 100 amino acid consensus (Figure V.5), it is clear that the fly protein is very distantly related to the four known UPRTases, with only 33% of the UPRTase-specific residues conserved. URK1, which is known *not* to contain a UPRTase activity, since it is unable to rescue mutations in FUR1 (the true yeast UPRTase) (Kern et al., 1990), has a score very similar to dART1, 27%. The scores of the known UPRTases range from 70% to 95%. While the molecular structure of UPRTase is not known, the cofactor binding sites, common to all the UPRTases, have been mapped (Andersen et al., 1992). These include the PRPP-binding site and the uracil-binding site, as indicated in Figure V.4. In addition to having a low UPRTase score, dART1 is missing both of these highly conserved regions. This leads to the conclusion that while dART1 is related to FUR1, and the other UPRTases, it is probable that it does *not* have a UPRTase activity.

Discussion

Using the yeast interaction trap system, dART1 was identified as a specific pseudophosphoNERD binding protein. No interaction with NERD was detected. Although I was unable to show *in vitro* that dART1 binds to phosphoNERD, the possibility remains that it does and this should be pursued vigorously. Ideally, the interaction should be tested with baculovirus-produced proteins, not bacterial proteins, but time did not permit this analysis.

The importance of this putative interaction is that GAP-43, and by analogy NERD, is thought to be activated by phosphorylation. Therefore the

name	activity	% UPRTase consensus	% dART
URKc	?(but not UPRTase)	27	27
STR	uracil + PRPP --> UMP	95	21
UPP	uracil + PRPP --> UMP	87	17
MYCH	uracil + PRPP --> UMP	89	18
FUR1	uracil + PRPP --> UMP	70	41
dART1	???	33	--

Figure V.5: dART1 is probably NOT a UPRTase

dART1 is poorly related to the UPRTase consensus defined in Figure V.4. The function of URKc is not known but it does not have a UPRTase activity. Since a 27% consensus score is insufficient for UPRTase activity in URKc, dART1 is also unlikely to have this function. This inference is strengthened because dART1 is also missing two important cofactor-binding sites, as is URKc. dART1 is unlikely to be a homolog of URKc since URKc is as closely related to dART1 as it is to the UPRTase consensus sequence.

downstream target of phosphoGAP-43 may be mediating the function of the protein.

What is the function of dART1? Based on sequence analysis alone, it is unlikely that dART1 is a homolog to the yeast UPRTase. Although there is a considerable primary amino acid sequence identity between the proteins, 41% within a 250 amino acid stretch, several highly conserved motifs common to all UPRTases is lacking. Mycoplasma, gram-positive bacteria, gram-negative bacteria and yeast are at least 3 billion years separated from each other evolutionarily (Knoll, 1992). It is fair to argue that relationships preserved between all four species should be highly conserved in *Drosophila*. This is not found to be the case when comparing dART1 with the UPRTase consensus. Most importantly, the conserved substrate binding sites have been lost. This implies that the function of dART1 has diverged.

Two recent papers suggest that the phosphorylated form of GAP-43 (Strittmatter et al., 1993) and neurogranin (Cohen et al., 1993) interact with a G protein-coupled inositol triphosphate pathway in *Xenopus* oocytes. Activation of the endogenous G protein system through receptor activation of acetylcholine receptors leads to phospholipase C activation, IP_3 production, release of calcium from intracellular stores, and finally, activation of calcium-sensitive chloride conductances. Microinjection of neurogranin RNA into oocytes and GAP-43 protein into oocytes lead to a potentiation of the evoked-chloride conductance. Cohen *et al.*, were able to show that the phosphorylation state of the serine in the GAP43 module was crucial in generating the effect: a serine-to-glycine mutation abolished the increased response and phorbol-ester specifically increased the response in cell injected with wildtype neurogranin RNA. While the requirement for phosphorylation in the GAP-43 experiment was not evaluated, it is very likely that both proteins work in the same manner. Neither experiment

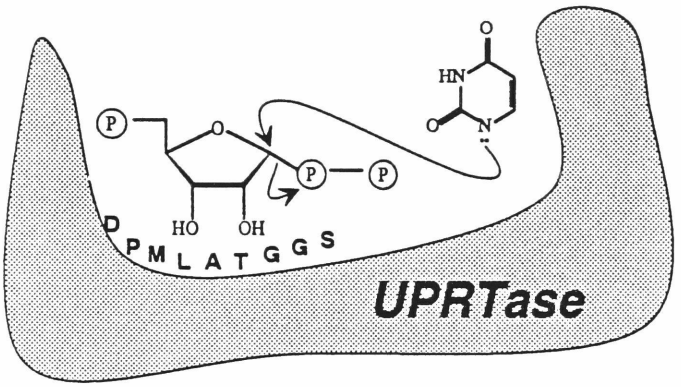
addressed which step in the G protein coupled response was the focus of GAP-43/neurogranin activity.

In the late 1970's, Moss and Vaughan (Moss and Vaughan, 1978) described a class of proteins that catalyzed the addition of ADP-ribose to acceptor proteins, much like the well-studied cholera and pertussis enterotoxins. These toxins enzymatically attach ADP-ribose to a class of G proteins and thereby alter their function. In particular, cholera toxin ADP-ribosylates a C-terminal arginine of the G protein and in doing so inhibits the protein's intrinsic GTPase activity, leading to a constitutively activated form of the protein (Van Dop et al., 1984). Pertussis toxin attaches ADP-ribose to a conserved C-terminal cysteine to the same G protein (Hurley et al., 1984). This modification leads to an opposite result, namely the inhibtion of G protein function, by interfering with receptor mediated GDP exchange. Moss went on to characterize a number of endogenous proteins with transferase activity, although the nature of the *in vivo* substrates has been difficult to resolve. What is clear however, is that these enzymes must be strictly regulated in order for G proteins to respond normallly to signalling information.

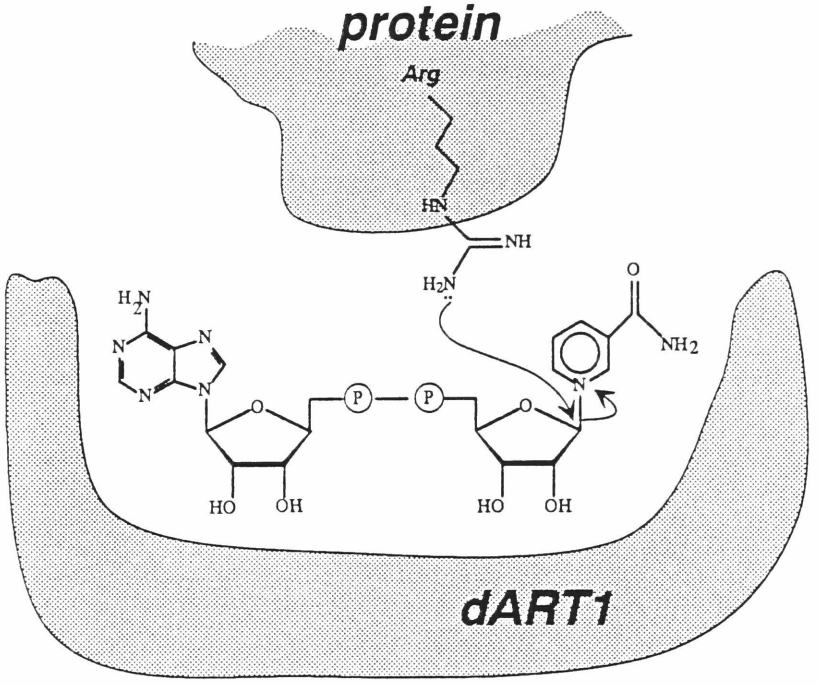
One characteristic of ADP-ribosyltransferases (ARTases) is that they are inhibited by uracil and uridine analogs (Banasik et al., 1992). Since the chemical structure of uracil is similar to nicotinamide (a product of ARTase) the mechanism of this inhibition may be a simple end-product poisoning. Since uridine is a five-fold more potent inhibitor than nicotinamide suggests that UPRTases and ARTases may have similar catalytic sites (see Figure V.6). The NAD:ARTase reaction involves the nucleophilic attack of a tertiary amine on the hemiacetal carbon of ribose with nicotinamide as the leaving group. UPRTases have a conceptually similar chemistry with the secondary ring amine of uracil attacking the same carbon atom and displacing pyrophophate in an S_N2 reaction.

Figure V.6: Author's conceptualization of dART1 activity

Cartoon of one possible function for dART1 as an endogenous ADP-ribosyltransferase (ARTase). The picture highlights the similar chemistry between UPRTase and ARTase. Although arginine is depicted as the ADP-ribose acceptor, histidine, lysine and asparagine are also known to be mono ADP-ribosylated *in vivo* (Ueda and Hayaishi, 1985). The sequence thought to be responsible for PRPP-binding, DPMLATGGS, was drawn under the PRPP substrate.



uracil + PRPP ⟶ UMP + PP_i



NAD + protein ⟶ ADP-ribose-protein + nicotinamide

I propose that dART1 may be an endogenous ARTase. Unfortunately, there are no reports of cloned ARTases for comparison. A model for GAP-43/NERD function based on this proposal is presented in the final chapter.

It is not uncommon for homologous proteins to evolve different enzymatic activities (Murzin, 1993). Methionyl aminopepeptidase (MAP) and creatinase share a similar folding motif in addition to a related amino acid sequence. However their substrates are quite different. Novel catalytic functions can be crafted through the evolution of a common structural framework. The consensus between URKc, the UPRTases and dART may define a labile enzymatic backbone.

Chapter VI: *nerd* genetics

Introduction

Access to a genetic analysis is the implicit promise of *Drosophila* biology. Apart from interesting glimpses at the evolution of protein structure, the primary impetus of studying fly homologs is fused with the hope of obtaining functional information through a genetic analysis. Examples of the successful application of reverse genetics abound in the literature, one of the most recent being the analysis of synaptotagmin in *Drosophila* (DiAntonio et al., 1993b).

The success of any screen for mutants is always dependent on the nature of the assay. In the attempt to find *nerd* mutants, I have assumed that the absence or alteration of NERD function will result in a lethal phenotype. This assumption is based on two pieces of data. First, the expression of *nerd* is tightly regulated during embryogenesis, suggesting (though not proving) that NERD-dependent functions are required there. Second, a null mutation in GAP-43 has recently been generated in the mouse (Mark C. Fishman, pers. comm.). Mice lacking GAP-43 are unable to feed and usually die within a short time following birth, indicating a *vital* function for GAP-43 in mammals. A number of laboratories have undertaken a screen for GAP-43-like molecules in the fly prior to this study (Ng et al., 1989)(J.B. Watson, pers.comm., M.Fishman, pers. comm., E. Chapman, pers. comm.) using PCR-based methods, immunological methods and DNA hybridization-based methods (as in this work) and have failed to find any convincing relatives. If *nerd* is the sole homolog of GAP-43 and the knockout mutation of GAP-43 is lethal in the mouse, it is reasonable to assume a lethal mutation in *nerd* can be generated.

In this chapter I detail the genetic characterization of the *nerd* locus. I have conducted a screen to recover lethal mutations in *nerd* using classical genetic techniques. I present evidence that the region of the chromosome where *nerd* lies

is approaching saturation for lethal complementation groups. One of these genes may be *nerd*.

Results

Figure VI.1 is a photomicrograph of an *in situ* hybridization of digoxigenin-labeled *nerd* DNA to the polytene chromosomes of the larval salivary glands. In Figure VI.1, upper panel, hybridization to wild-type chromosomes gave a unique signal from position 51D/E on the the right arm of the second chromosome (Bridges, 1935). 51D/E is a fairly well-characterized region with several available deficiencies (Underwood et al., 1990). In Figure V1.1, lower panel, an *in situ* with the same probe as in the upper panel was hybridized to heterozygotes with the genotype Df(2R)XTE11/cn bw. The deficiency chromosome XTE11 deletes a large interval in 51D/E. As the picture clearly shows, the probe hybridizes to only half of the chromosome, indicating that XTE11 deletes *nerd*.

Since XTE11 deletes *nerd*, this chromosome is sufficient as a "tester" chromosome to induce point mutations in the *nerd* locus. The schematic for generating these mutations is outlined in Figure VI.2. Briefly, chemically mutagenized *cnbw* flies are crossed *en masse* to virgin females. From this cross, single male animals that contain a mutagenized second chrmosome, *cnbw** (the asterisk denotes a mutagenized chromosome), balanced with CyO are crossed to females carrying the XTE11 deficiency. Males that contain a lethal mutation in the XTE11 (*nerd*) region will not produce non-balancer offspring (in this example, all flies from such a cross will have curly wings). Eight thousand single pair-matings were conducted in this fashion, and twenty-six lethal mutations were recovered. These mutants were crossed *inter se* to establish lethal complementation groups. The twenty-six mutants mapped to six lethal genes in

Figure VI.1: *nerd* localizes to the right arm of the second chromosome at position 51E

In the upper panel, a *nerd* genomic probe has been labeled with digoxigenin and hybridized to the polytene salivary glands of a wildtype fly. The glands were counterstained with Giemsa to visualize the polytene banding pattern. A single band (arrowhead) was seen at 51E. In the lower panel, the same probe was hybridized to glands heterozygous for the deficiency Df(2R)XTE11, which removes cytological bands 51E3;52A10. Note that in this *in situ* only half the chromosome hybridizes to the probe, indicating that in the XTE11 deficiency , *nerd* is absent.

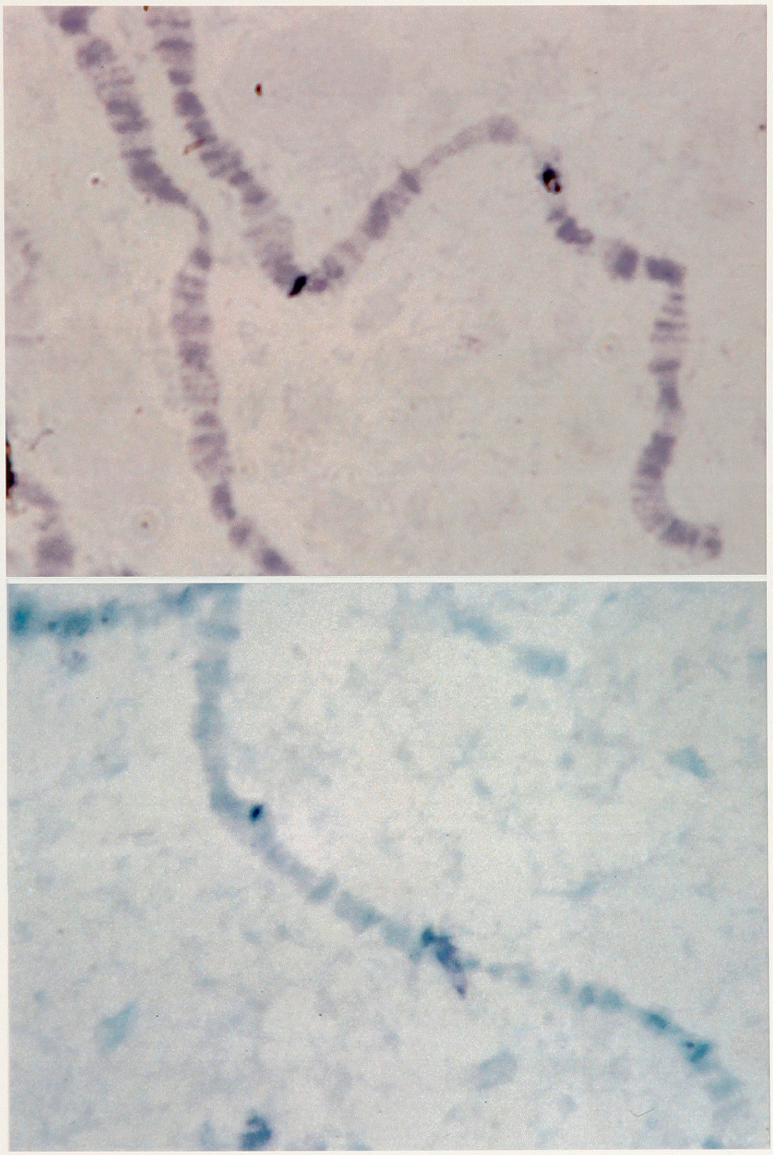
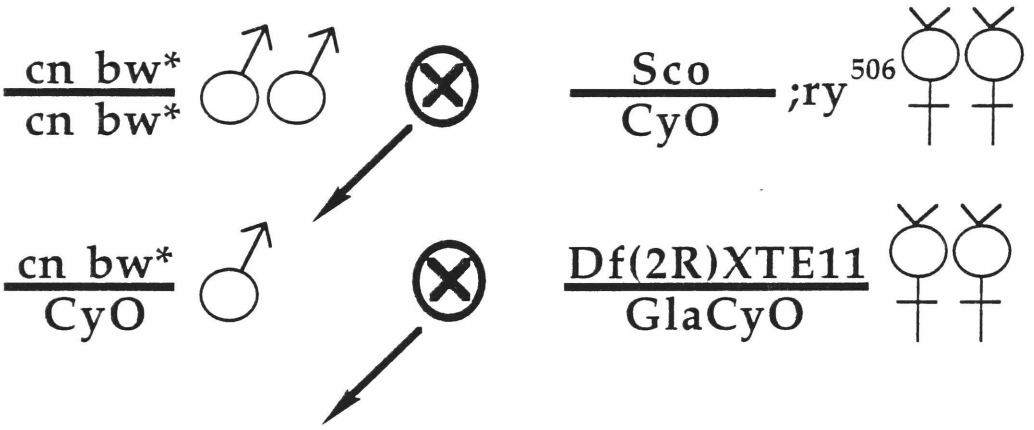


Figure VI.2: Outline of the mutagenesis screen to find lethal *nerd* mutations

An isogenized *cinnabar brown* (*cn bw*) stock was mutagenized as described in Materials and Methods. Males were crossed *en masse* to virgin females of the genotype Sco/Cyo;ry^{506} at a ratio of 4 females for every male. 7,600 single male progeny of the genotype *cn bw**/CyO were individually mated to 5 virgins of the phenotype Df(2R)XTE11/GlaCyO. Pair matings which failed to produce progeny of the genotype *cnbw**/Df(2R)XTE11 were maintained as balanced stocks and scored as lethal mutations in XTE11. Twenty-six lethal mutations were isolated in this fashion.

Generation of EMS-induced lethals at the *nerd* locus



select lines where cn bw*/Df (2R)XTE11 is lethal

7800 IInd chromosomes screened
→ 26 lethals (0.3%)

XTE11. This number of lethals is quite low considering the size of the XTE11 deficiency. However, the region has been studied by other groups interested in the very large number of female-sterile (and hence **not** lethal) genes located there (Underwood et al., 1990). Moreover, in association with another laboratory (E. Underwood, Bowling Green State University) we have increased the number of screened chromosomes to 45,000. The number of lethal complementation groups (genes) picked up in the combined screen has remained at six. Given the size of the deficiency target and the the number of flies screened it is possible that we have reached saturation of the region for lethal mutations. If *nerd* mutations **can** lead to lethality, it is likely that one of the identified genes in the region is *nerd*.

In order to clarify which mutants were potential *nerd* mutants, the *nerd* gene was mapped more finely with other deficiencies in the region. This mapping was achieved by use of a "deficiency Southern." In this experiment (Figure VI.3) various deficiencies in the *nerd* region are crossed to a laboratory strain (A24) that shows a natural restriction fragment length polymorphism (RFLP) in a BamHI digested Southern hybridized with 3' *nerd* DNA probe (~8.2kb fragment). The balancer chromosomes (GlaCyO) carry the wild-type restriction sites and show a larger fragment hybridizing (~9.0kb). Deficiencies are made heterozygous with either A24 or GlaCyO and DNA from these flies is digested with BamHI, run on a gel, transferred and probed with 3'*nerd* DNA. Deficiencies that uncover *nerd* will show only the A24 RFLP *or* the wild-type band (GlaCyO or their respective parental band, see XTE58) but not *both*. Deficiencies that do *not* delete *nerd* will show two bands when crossed to A24, the A24 RFLP *and* the parental RFLP. For example, A24/A24 (wild-type lab stock) shows a single 8.2kb fragment. A24/GlaCyO shows two bands, the smaller A24-specific RFLP and the larger GlaCyO RFLP. Df(2R)XTE11, which deletes *nerd* as shown in Figure VI.1, displays the A24 RFLP when heterozygous

Figure VI.3: The 3' end of *nerd* is localized to a small region in 51E defined by three overlapping deficiencies

In order to localize *nerd* more precisely, a deficiency southern was performed as descibed in the text. Briefly, a novel BamHI RFLP was discovered in a laboratory stock (A24) which produced an 8kb restriction fragment when hybridized with the 3' end of *nerd*. The GlaCyO chromosome has an 8.6kb fragment, and both fragments are seen in the heterozygous A24/GlaCyO animal, demonstrating that each chromosome has a copy of *nerd*. Each of three deficiences were crossed to both A24 and GlaCyO chromosomes to produce heterozygotes, and DNA was prepared from each of these genotypes. The deficiency XTE11, which by *in situ* analysis is known to be missing *nerd*, displays the 8.0kb RFLP when *in trans* with A24 and the 8.6kb RFLP when *in trans* with GlaCyO, confirming that XTE11 deletes *nerd*. Similar analysis indicates that the 3' *nerd* probe is absent in Df(2R)ℓ4 and present in both Df(2R)XTE58 and Df(2R)X-9. Therefore the structural gene for *nerd* lies between the distal XTE58 breakpoint and the proximal X-9 breakpoint (see Figure VI.4).

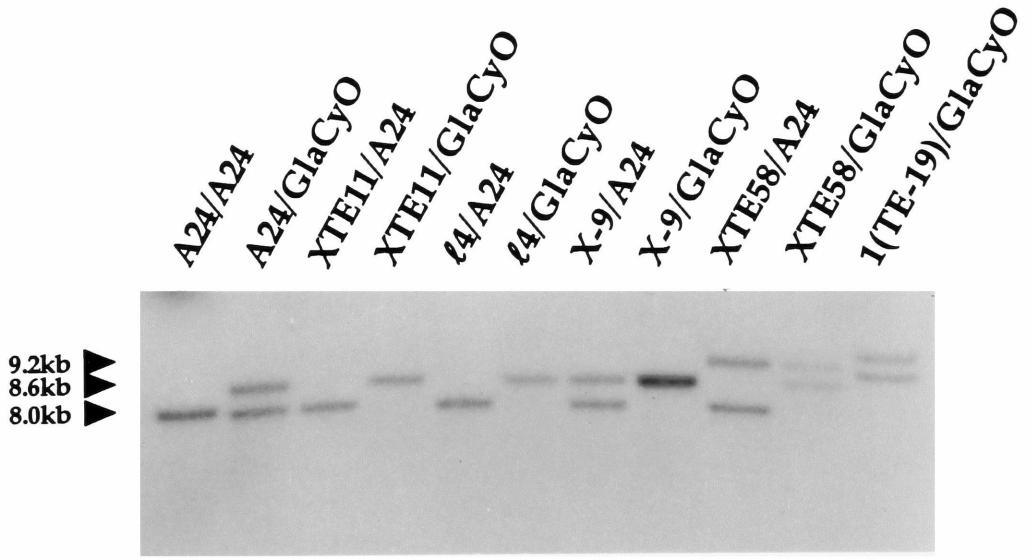
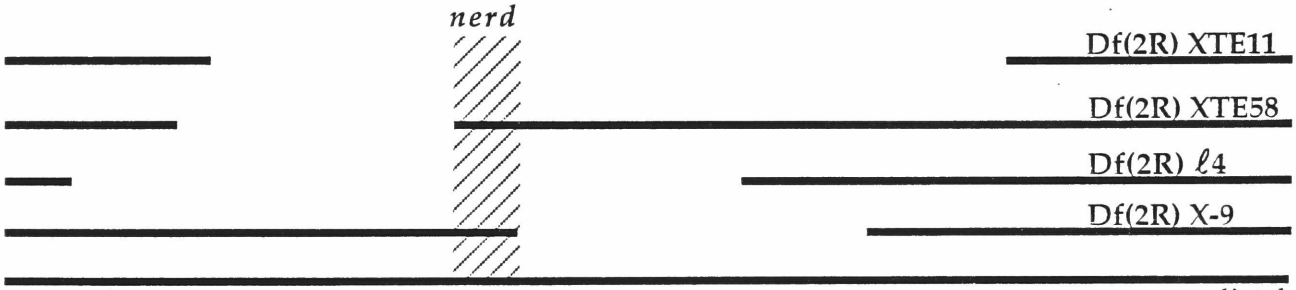

A24/A24
A24/GlaCyO
XTE11/A24
XTE11/GlaCyO
ℓ4/A24
ℓ4/GlaCyO
X-9/A24
X-9/GlaCyO
XTE58/A24
XTE58/GlaCyO
1(TE-19)/GlaCyO
9.2kb
8.6kb
8.0kb

Figure VI.4: Genetic map of the *nerd* locus

The mutants obtained from the mutagenic screen were crossed *inter se* and to the other deficiencies in region. The hatches region indicates the physical location of the 3' end of *nerd* as described in the text and Figure VI.3. Deficiencies are represented schematically by gaps in the solid line. Asterisks by allele numbers are stocks contributed by E. Underwood of Bowling Green State University.



W40	*LT2*	*P85*	*scab*	*D25*	*LT5*
W40	LT2	P85	N11	D25	LT5
S34	X2929*	MB2*	H102	P34	X-2*
			LT3	Q63	ℓ5*
			LT11	F44	
				Y28	
				LT1	
				LT4	
				LT7	
				LT8	
				HK4	

with A24 and the GlaCyO RFLP when heterozygous with GlaCyO proving that the XTE11 chromosome itself contains no *nerd* 3' DNA. The deficiency Df(2R)X-9 chromosome is an example of a deficiency in the vicinity of *nerd* that does *not* delete the 3' *nerd* DNA. X-9/A24 flies have *two* bands, the A24 RFLP and the X-9 RFLP. By this analysis, the 3'*nerd* DNA is shown to be deleted in Df(2R)*ℓ*4 and Df(2R)XTE11 and not deleted in Df(2R)X-9 and Df(2R)XTE58. This place the 3' end of *nerd* in a very small cytogenetic window, as shown in Figure VI.4.

All of the point mutants described above were crossed to the four deficiencies to generate a genetic map of the region. The lethal mutation referred to as *LT2* is closley linked to *nerd* since it displays the same association with the deficiencies as the *nerd* DNA does. That is, in deficiencies that delete the 3' *nerd* DNA probe (XTE11 and *ℓ*4) *LT2* is lethal. *LT2* is viable over deficiencies that do not delete the *nerd* DNA probe (XTE58 and X-9). Because of this tight correlation between the *nerd* gene and the *LT2* complementation group, it is possible that *LT2* represents a lethal mutation in *nerd*.

In a crude attempt to demonstrate that one of the chemically-induced lethal mutations was an alteration in *nerd*, I placed the *nerd-S* and *nerd-L* cDNA in the P-element transformation vector pCaSpeR-hs (Pirrotta et al., 1985). This vector carries the mini *white* gene to score transformation. Flies were transformed by microinjection (Rubin and Spradling, 1983) in the presence of helper transposase (Karess and Rubin, 1984). These constructs expressed *nerd* RNA when pulsed with heatshock (data not shown). P-element carrying lines were established in Df(2R)XTE11 backgrounds and the putative mutants were crossed in. None of the lines became viable in the presence of P[hs-NERD] constructs, including the gene tightly-linked to *nerd*, *LT2*. (data not shown).

Discussion

In this chapter I have presented the results of a mutagenesis experiment designed to isolate mutations at the *nerd* locus. *nerd* localizes to the right arm of the second chromosome in a region that has been characterized previously. In that study, ionizing radiation was used as the mutagen. Typically, gamma and X-ray irradiation produce deficiencies of varying size. Mutations produced in this manner are often detectable on Southern analysis. Therefore, in an initial screen for *nerd* mutants from the existing pool of mutants in the region I used full-length cDNAs as probes to detect any polymorphisms that might be associated with DNA rearrangements or deletions. None were detected (data not shown).

Because Underwood *et al.* were unable to meet the statistical requirements for saturation mutagenesis using the truncated Poisson distribution (Barrett, 1980), I extended the screen for lethals in the region. After screening an additional 8,000 chromosomes, I was able to increase by two the number of complementation groups in the relevant region, from 3 to 5. Subsequently, the group of Underwood has also extended the mutagenesis and have tested an additional 30,000 mutagenized lines, bringing the total number of chromosomes screened for lethal mutations in 51E to 48,000. The number of mutants in each complementation group has increased substantially

The gene *LT2* had been a promising candidate for *nerd* because it maps to a very small interval that lies between the distal breakpoint of XTE58 and the proximal breakpoint of X-9. This is precisely the region where the 3'*nerd* exon has been mapped by "deficiency Southern."

Snce the genomic structure, including the position of enhancers, has not been fully characterized, it is impossible to know if X-9 deletes important *nerd* sequences. Thus although LT2 is tightly linked to *nerd*, it is currently impossible

to discard some of the other loci between the distal XTE58 breakpoint and the distal ℓ4 breakpoint as potential *nerd* mutants. One locus, *scab*, can be ruled out on the basis of its phenotype. The *scab* gene specifically effects dorsal closure and leaves the CNS intact (Ian Dawson, personal communication). The size of the ℓ12 complementation group, 39 members, suggests that it is a very large gene, and thus unlikely to be *nerd*.. Several P-elements have been mapped to ℓ12 and none of these insertions have been detected by Southern (data not shown), another indication suggesting *nerd* is not ℓ12.

In addition to LT2, *P85* and the P-element-induced mutation MB-2 (Underwood, pers. comm.) seem to be viable candidates for *nerd* . Given the likely importance of GAP-43/NERD function in both the developing and adult nervous system, the identification of a mutation in the fly homolog of GAP-43 should be pursued vigorously. A more complete map of the genomic structure and the creation of a genomic *nerd* transgene will facilitate this undertaking.

Chapter VII: Summary and Discussion

Summary

This study has identified a neuron-specific gene in *Drosophila melanogaster*, *nerd*, based on its homology to the vertebrate GAP-43 gene. *nerd* encodes two overlapping proteins, NERD-L and NERD-S, both of which contain repeats of the GAP-43-related homology domain, the GAP43 module. Biochemical analysis of the *nerd* gene products revealed a number of similarities with its vertebrate cousin. The GAP43 S-module, found in both NERD proteins, is a PKC substrate and interacts with calmodulin. Two other GAP43-like modules, the V- and T-modules, also interact with calmodulin, but are not phosphorylated by PKC. Mutations mimicking phosphorylation by PKC disrupt the calmodulin interaction. Both NERD proteins have an anomalous migratory behavior on SDS-gels and are soluble in 2.5% perchloric acid, the latter an indication of their extreme solubility. While regions outside of the GAP43 modules are not conserved between species, the amino acid composition is similar, containing mainly alanine, glycine, proline and glutamate, and lacking in aromatic and hydrophobic residues. While GAP-43 is palmitoylated, NERD-L is myristoylated *in vitro* on glycine. This co-translational modification may assist the protein in either subcellular targeting of membrane-binding, as palmitoylation does for GAP-43.

In situ localization of both the *nerd* transcript and the NERD protein reveals neuron-specific patterns of expression in the developing embryo. The temporal distribution of NERD suggests that the protein is not required for early neuritogenesis, as had been proposed for GAP-43 based on a very early expression pattern in vertebrate development. The timing of NERD expression is coincident with late axonal outgrowth and synaptogenesis.

A potential specific-target for the phosphorylated GAP43 module, dART1, was identified in the yeast two-hybrid trap system. Although the function of dART1 is unknown, regions of strong homology to the yeast uracil phosphoribosyltransferase enzyme suggest it may have a related enzymatic function. ADP-ribosyltransferase activity was suggested as a potential activity.

A genetic analysis of *nerd* was initiated and a lethal complementation map of genes mapping at or near the *nerd* locus was assembled from a mutagenic screen conducted in the laboratory. Of approximately 8000 second chromosomes tested, 26 lethals in 6 complementation groups were identified. *nerd* maps to a very small deficiency that uncovers only one of the six lethal genes, *LT2*. It has not been firmly concluded whether *LT2* is a lethal allele of *nerd*, although supplying the animals with the wildtype NERD product (NERD-L or NERD-S) through inducible heatshock-NERD transgenes did not rescue the lethality associated with the *LT2* mutation.

Discussion: A Unified Theory for GAP-43 Function?

GAP-43 is an intriguing molecule. Its conspicuous presence in either axonal outgrowth, regeneration or synaptic plasticity would make it interesting, but its simultaneous presence in all three behaviors makes it particularly interesting. An attempt to synthesize a putative role for GAP-43 from the diverse investigations that been conducted in the last 20 years should, I believe, make the reductionist's assumption that GAP-43 is doing the same thing whenever and wherever it is found. It is conceivable that it plays more than a single role - perhaps a passive, structural role and an active, signalling role - but for the purpose of this discussion I will propose a single active role for the molecule based on the interaction of pseudophospho-NERD with dART1, a presumptive ARTase.

Is there any evidence that ARTases play an important role in the brain? If GAP-43 function is tied to an ARTase, at the very least the expression of the proteins should be coupled. Recently, Duman *et al.* (Duman et al., 1991) have attempted to characterize the brain ARTases and their natural substrates in hippocampal tissue. The ARTase enzymes are abundant in the brain. Curiously, one of the ADP-ribosylated proteins they found was a 50kD protein whose identity was defined by Coggins *et al.* to be GAP-43 (Coggins et al., 1993a). The same group has provided evidence that neurogranin is also ADP-ribosylated (Coggins et al., 1993b). Since GAP-43 and neurogranin only share the GAP43 module in common, it is probable that the conserved module, in addition to the other myriad functions associated with it, is the target for the transferase activity.

Do brain transferases have a role in neuronal behaviors? There are two relevant reports addressing this issue. Duman *et al.* (Duman et al., 1993) have shown that there are alterations in the overall level of ADP-ribosylation in hippocampal slices after the induction of LTP. They were careful not to indulge too heavily in an interpretation of the results. Although they found *decreased* levels of ADP-ribosylated GAP-43 *in vitro* following LTP, they could not ascertain whether this was the result of an increased *in vivo* ARTase activity that would block their *in vitro* assay (by ribosylating GAP-43 during LTP), or whether LTP reduced the overall ARTase activity in their *in vitro* extracts. The possibility that LTP induces a transient increase in ARTase activity was not directly addressed. The only result to be drawn from these experiments is that *altered* levels of ARTases are found in association with LTP. A strong piece of evidence from D.V. Madison's laboratory is that specific inhibitors of ARTases, for example vitamin K_1, block LTP formation in the hippocampus (Schuman et al., 1992). This is direct evidence that ADP-ribosylation, in some form, normally occurs during the formation of LTP.

Thus, there is an indication that ARTases in the brain interact with GAP-43-like molecules and that ARTase activation has a role in establishing LTP. Because G proteins have been shown to be involved in a number of important neuronal behaviors, the presence of an endogenous G protein modifying system would have many ramifications. A number of neurotransmitters have autoreceptors on growth cones and presynaptic membranes. The activation of these receptors often leads to the inhibition of voltage-dependent calcium channels, although some have been found to increase potassium current (Wilk-Blaszczak et al., 1994). Inhibition of calcium current (and therefore decreased intracellular calcium) and activation of potassium current (causing membrane hyperpolarization) could have similar effects on neurotransmitter release. Examples of transmitters known to affect calcium currents directly are norepinephrine (Forscher et al., 1986), glutamate (Swartz and Bean, 1992), luteinizing hormone releasing hormone (LHRH) (Boland and Bean, 1993), $GABA_B$ (Grassi and Lux, 1989), adenosine (Kasai and Aosaki, 1989) and acetylcholine (Beech et al., 1992). Where it has been investigated, this inhibition is mediated by pertussis-sensitive G proteins, probably of the G_o subtype (Kleuss et al., 1991). Neuroblastoma NG 108-15 cells express receptors for at least four different neurotransmitters (leu-enkephalin, norepinephrine, somatostatin and bradykinin), all of which inhibit the N-type calcium channel through a agonist-specific G protein (Taussig et al., 1992). In *Aplysia* as well, G proteins have been shown to inhibit transmitter release at an identified cholinergic synapse (Baux and Tauc, 1991). G proteins are tonically active in the absence of transmitter receptor stimulation and depress basal calcium currents (Kasai, 1991). G_o has been co-purified with N-type calcium channels and therefore the inhibition of channel function is believed to be through a direct protein-protein interaction (McEnery et al., 1994) that affects the voltage-sensitivity of the

channel. One way G proteins may inhibit N-type calcium channel activity is by altering calcium ion permeation (Kuo and Bean, 1993).

In a remarkable study, Swartz (Swartz et al., 1993) showed that the activation of PKC could disrupt both the transmitter-induced inhibition and the tonic inhibition of G proteins in acutely dissociated CA3 hippocampal neurons. He proposed several possible PKC targets that could mediate the effect. Phosphorylation of the calcium channel or one of its regulatory subunits could be the locus of the effect , since purified α1-subunit can be phosphorylated *in vitro* (Ahlijanian et al., 1991). G protein inactivation by direct phosphorylation could be another, as scattered reports have appeared that some subtypes of G proteins are PKC substrates (Zick et al., 1986).

Since stimulation of PKC with phorbol esters facilitates synaptic transmission (Malenka et al., 1986), the detailed mechanism that Swartz elucidates has important implications for the modulation of presynaptic neurotransmitter release and therefore synaptic plasticity. It seems reasonable to suggest that GAP-43, a major presynaptic PKC substrate could be involved in PKC-mediated events that involve the calcium channel.

Because many of the components of the presynaptic membrane are present in the growth cone, similar mechanisms of calcium control may prevail. The growth cone cytoarchitecture is extremely sensitive to calcium levels (Mills and Kater, 1990). Calcium-sensitive actin-binding proteins, for example α-actinin, are concentrated in filopodia and may mediate motility or adhesion (Sobue and Kanda, 1989). G proteins, particularly the N-type calcium channel-binding protein G_o, are present in very high concentrations in growth cone particles (Strittmatter et al., 1990). GTPγ-S, which constitutively activates G proteins has an inhibitory effect on neurite outgrowth of sympathetic neurons, whereas GDPβ-S, which inhibits G protein function, has a stimulatory effect on

the same neurons (Strittmatter et al., 1992). Similarly, neurite outgrowth in PC12 cells grown on monolayers of cells transfected with cell-adhesion molecules (like L1) can be fully inhibited by pertussis toxin and substantially inhibited by N-type calcium channel blockers (Williams et al., 1992). Protein kinase C has been shown to regulate outgrowth on laminin, another cell-adhesion molecule (Bixby, 1989). In many ways, the same molecular players seemed to be involved with setting the calcium level in both the presynaptic membrane and the growth cone. GAP-43 expression, particularly phosphoGAP-43 expression (Meiri et al., 1991), is largely confined to these precise domains.

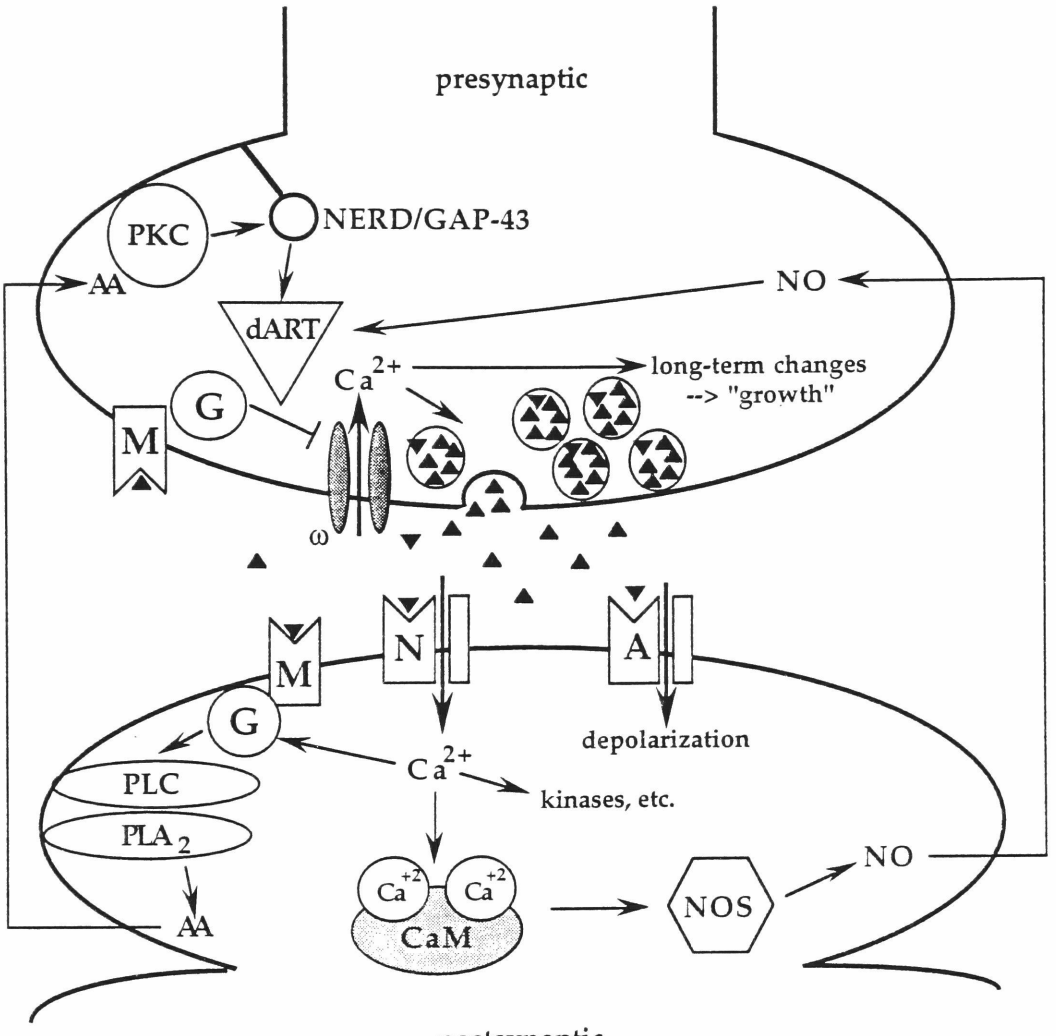
I propose the following heuristic model (see Figure VII.1) which incorporates the proposal that dART1 may be an endogenous ARTase into a scheme that may modulate calcium channel activation. At a "plastic" synapse, depolarization of the presynaptic membrane causes neurotransmitter release. Normally, autoreceptors for the released transmitter cause a feedback inhibition of the presynaptic calcium channels via a receptor-linked G protein. Tonic activation of the presynaptic membrane would therefore lead to a decrease in calcium current over time and a decreased amount of neurotransmitter release with a given impulse, leading to desensitization. However, if the stimulation is sufficient to induce postsynaptic LTP, for instance in the presence of tetanic stimulation or paired input, retrograde messengers from the postsynaptic cell would be liberated and travel back to the presynaptic membrane to activate PKC. PKC then phosphorylates GAP-43. PhosphoGAP-43 activates dART1 which ADP-ribosylates the inhibitory G proteins, causing a disinhibition of N-type

Figure VII.1: The Model

A schematic for GAP-43/NERD function based on Bliss and Collingridge (Bliss and Collingridge, 1993). A presynaptic impulse depolarizes the membrane and voltage sensitive calcium channels (ω) are activated, leading to calcium influx and subsequent L-glutamate release. In the absence of postsynaptic activation, the presynaptic autoreceptors for glutamate (M) are stimulated and G-proteins are activated leading to inhibition of calcium channels. This process causes a decrease in presynaptic basal calcium levels and decreased calcium influx during subsequent presynaptic depolarizations.

However, if the postsynaptic membrane is activated, as a result of either multiple simulataneous inputs or tetanus, ionotropic AMPA glutamate receptors (A) are activated and the postsynaptic membrane is depolarized, releasing the magnesium block on the NMDA receptors (N), which leads to calcium influx postsynaptically. In addition metabotropic (M) glutamate receptors coupled to phospholipase C are turned on Retrograde messengers are released (perhaps arachidonic acid(AA) or nitric oxide(NO)) which signal to the presynaptic membrane. Protein kinase C (PKC) is activated and NERD/GAP-43 is phosphorylated. PhosphoNERD then activates dART1, leading to G-protein inhibition in this scenario. The net result of this chain of events is a transient increase in calcium channel activity. Alternatively, dART activation might alter other cellular functions including actin polymerization. See text for more details.

Phosphorylated NERD/GAP-43 may be involved in establishing long-term presynaptic alterations



adapted from Bliss, 1993

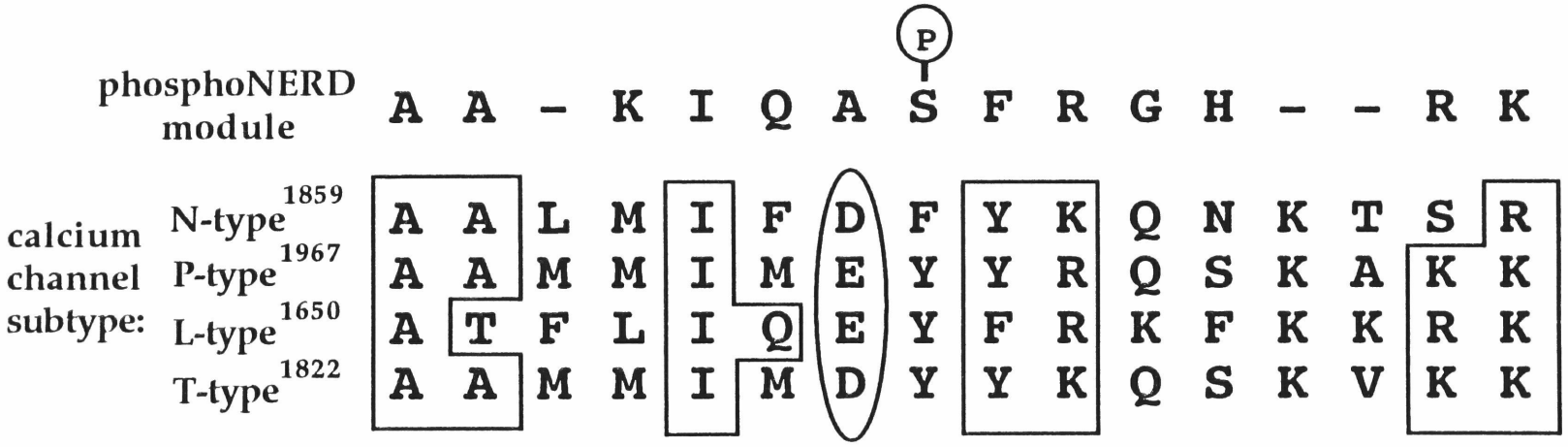
calcium channels. As an interesting aside, there may be a dART-binding domain resident in voltage-sensitive calcium channels (Figure VII.2). If dART activity takes place in the proximity of the these channels, a low affinity binding site might serve to concentrate the enzyme where it would ultimately be called on to function. This potential dART-binding region has an IQ motif, which is recognized as a common domain mediating protein-protein interactions. However it is unusual in that the channel IQ domains are the only IQ domains identified to date that have acidic residues in proximity to the canonical GAP-43 PKC-sites.

Inactivation of G proteins could affect many other cellular behaviors apart from calcium channel activation. Scholz and Miller (Scholz and Miller, 1991) have shown that activation of the presynaptic adenosine receptor inhibits neurotransmitter release through a pertussis-sensitive G protein which is not linked to a calcium channel. They propose that a distinct G protein pathway affects the vesicle fusion apparatus which mediates neurotransmitter release. This alternate route to neurotransmitter release might explain recent results (Cormier et al., 1993) suggesting that LTP can be induced in the absence of presynaptic activity.

The generation of LTP is a complicated phenomenon and involves the coordination of pre- and postsynaptic mechanisms (Malenka and Nicoll, 1993). Since the phosphorylation of GAP-43 seems to tightly correlate with this process (Gianotti et al., 1992), the temporal interaction of phosphoGAP-43 and dART may be involved. Leahy *et al.* (Leahy et al., 1993) have recently shown *in vivo* that presynaptic PKC activation and, consequently, GAP-43 phosphorylation occur following LTP-producing tetanic stimulation. Inhibitors of the NMDA receptor (AP5), block LTP and presynaptic PKC-mediated phosphorylation of GAP-43, again suggesting the presence of a retrograde message directing GAP-43

Figure VII.2: Voltage-sensitive calcium channels have a pseudophosphoNERD-like motif

Initial databank searches with a degenerate GAP43 module sequence indicated that L-type (dihydropyridine-sensitive) calcium channels have an 'IQ' motif. When other voltage -sensitive channels were retrieved a similar sequence in the C-terminal domain was revealed in all known classes. The acidic residue in the position just N-terminal to the traditional GAP43 phosphoserine is always conserved. In general the different calcium channels are very different from one another in the C-terminal region. Although no evidence is presented here, it is interesting to speculate that this region may represent a low affinity dART-binding site. Such a site might serve to localize dART, without activating it, in the vicinity where it will eventually be called on to function.


P
phosphoNERD module
A A - K I Q A S F R G H - - R K
calcium channel subtype:
N-type 1859
A A L M I F D F Y K Q N K T S R
P-type 1967
A A M M I M E Y Y R Q S K A K K
L-type 1650
A T F L I Q E Y F R K F K K R K
T-type 1822
A A M M I M D Y Y K Q S K V K K

activation. A G protein modification system would add another level of regulation to modify the neuron's complex repetoire of behaviors.

A simple, though indirect, test of my hypothesis might be assessed using Swartz's model system. If an ARTase (dART1) is interposed between PKC activation and G protein inhibition, concommitant activation of PKC (by phorbol ester) and *inhibition* of ARTase function (by vitamin K1) should block PKC activity, that is reverse the G protein inhibition mediated by PKC.

GAP-43's dynamic presence during so many important neuronal processes must indicate an important role for the protein. Unfortunately, progress in the field has been impeded by the lack of a genetic analysis of the protein's function *in vivo*. With the discovery of NERD in the developing and adult nervous of *Drosophila melanogaster*, such an analysis will be possible shortly. While I am personally frustrated that a *nerd* mutation was not defined in this study I am very confident that one either exists in the current collection of lethal mutations or will shortly. Understanding GAP-43 function will not only provide insights into developmental neurobiology and the mechanisms that underlie learning and memory, but will permit a more thorough investigation of the pathology behind the many devastating degenerative neurological diseases that afflict humankind.

Materials and Methods

Genetics and EMS mutagenesis

Fly stocks were raised on standard cornmeal-yeast-agar medium and maintained at room temperature or 18°C for storage. The following lines were obtained from Eileen Underwood (Underwood et al., 1990) at Bowling Green University.

Df(2R)ℓ4/GlaCyO

Df(2R)X-9/SM6B

scab alleles: ℓK4/GlaCyO, ℓ25/CyO

ℓ(2)51Eb alleles: ℓ12/CyO, ℓA/CyO, ℓ152/CyO

ℓ5/CyO

ℓ13/CyO

Df(2R)XTE11/GlaCyO and Df(2R)XTE58/GlaCyO were generated by M.B. Davis (Davis and MacIntyre, 1988) and are deletions of the transposable element line *y,w,rst*;1(TE-19)/GlaCyO.

AS785/CyO is a ry^+ P-element induced *scab* allele produced in Allan Spradling's laboratory and was obtained from K. Matthews at the Indiana University(IU) Drosophila Stock Center. w^a,*fas*; Df(2R)Jp4/CyO was also obtained from the IU Stock Center and is the deletion 51F13;52F8-9.

Df(2R)XTE11/GlaCyO deletes the *nerd* locus (see text) and was used as a tester chromosome in order to isolate potential *nerd* point mutations. Laboratory stocks of *cinnabar*(*cn*) and *cinnabar brown*(*cnbw*) were isogenized prior to mutagenesis. Twenty-five millimolar ethylmethane sulfonate (Sigma) was used as a mutagen and fed overnight to 3-4 day old *cn* or *cnbw* males in a solution of 1% sucrose/10mM Tris•HCl,pH 7.5. These males were crossed *en masse* to *Sco*/CyO;ry^{506} or Df(2R)XTE11/GlaCyO virgin females. Single F_1 males of the genotype *cn*(or *cnbw*)*/CyO(orGlaCyO) were mated to Df(2R)XTE11/GlaCyO virgin females. Lines that produced no *cn*(or *cnbw*)/Df(2R)XTE11 animals were

maintained as balanced stocks with the GlaCyO balancer. Complementation group assignment was made by crossing the lethal lines *inter se* and to identified lethals in the region (Underwood et al., 1990). *cnA24* is a lethal mutation that lies outside of XTE11 and was used for RFLP analysis(see text).

Cytological breakpoint for the deficiencies used in this work are as follows:

Df(2R)XTE11 : 51D3-E3-4; 52A6-10

Df(2R)XTE58 : 51D1-2; 51E5

Df(2R)ℓ4 : 51C3-7; 51E7-11

In situ ***Chromosome Localization and Deficiency Analysis***

Either OregonR (wild-type) flies or deficiency stocks outcrossed to OregonR were used to produce third-instar larvae. The salivary glands from these animals were dissected in lactic acid/acetic acid and squashed and fixed onto gelatinized slides (Ashburner, 1989). A 2kb SacI genomic fragment from the phage B3 was nick-translated with 16-dUTP (ENZO Biochem) and hybridized to the chromosomes in aqueous solution at 58°C for 16 hours. The slides were washed 3 times in 2xSSC at 45°C prior to signal detection with a streptavidin-linked peroxidase complex (DeTek kit, ENZO Biochem) and diaminobenzidine. The chromosomes were counterstained in a 1:20 dilution of Giemsa stain in 10mM sodium phosphate, pH 6.8 and examined using phase-contrast optics.

nerd Cloning and Northern Analysis

Two degenerate oligonucleotides of the following sequence were used in a PCR reaction with the rat GAP-43 cDNA GAPECO (Karns, et al., 1987):

A2: 5'-GGACTCGAG(C/T)T(A/T/C/G)TG(T/C)TG(T/C)AT(A/T/C/G)(A/C)
G(A/T/C/G)(A/C)G(A/T/C/G)AC-3'

GP: 5'-AGGGAATCGATC(G/T)(A/T/C/G)GT(A/T/G)AT(A/G)TG(A/T/C/G)

CC(A/T/C/G)C(T/G)(A/G)A-3'

The resulting 161bp fragment was gel purified, end-labeled with [γ-^{32}P]ATP (3000Ci/mmol, Amersham), and used in a low-stringency hybridization (25% formamide, 6xSSPE, 100µg/ml denatured salmon sperm DNA, 0.1% SDS and 5xDenhardt's) at 37°C overnight with a commercially obtained *Drosophila* genomic library (Promega). Approximately 10^5 plaques were screened on nylon filters. The filters were washed for 20 minutes at 37°C (2xSSPE, 0.1%SDS) twice, and once at 50°C for 10 minutes. The filters were exposed for one week and hybridizing phage were rescreened to homogeneity. One hybridizing clone was selected (B3) and phage DNA was prepared using standard methods (Sambrook et al., 1989). A 2kb SacI fragment containing all of the hybridizing sequence was subcloned into pGEM-7 (Promega) and sequenced using Sequenase and the manufacturer's instructions (US Biochemical). This fragment was used to screen a pupal cDNA library prepared in λZAPII (Stratagene) by Simon Kidd, and several hybridizing phage were isolated and partially sequenced. Two clones, pZd6 and pZd10, were selected for further analysis. Poly(A)+ RNA from various developmental stages was isolated, blotted to nylon and hybridized to a nick-translated B3 SacI fragment by Simon Kidd as described (Kidd, 1992).

In Situ *Hybridization to* Drosophila *Embryos*

Oregon R embryos were collected in population cages and staged (Campos-Ortega and Hartenstein, 1985). They were fixed and probed with a digoxigenin labeled B3 SacI subclone using the method of Tautz (Tautz and Pfeifle, 1989) and processed as described in Corbin *et al.* (Corbin et al., 1991).

PKC Experiments

pGEX-2T, a glutathione transferase fusion vector (Pharmacia), was modified to include a SacI site by cleaving the parent vector with EcoRI, generating blunt ends with Klenow polymerase and adding SacI linkers. SacI digestion, gel purification and religation gave the plasmid pGEX-2T(Sac7). This vector was digested with XmaI, filled-in with Klenow polymerase, cleaved with SacI and phosphatase treated. NERD-L cDNA (pZd6) in pBluescript was cut with SacII, blunt-ended with T4 polymerase , gel-purified, digested with SacI and directionally ligated into pGEX-2T(Sac7). In-frame fusions with GST were confirmed by sequencing. This manipulation yielded a GST:NERD-L fusion including amino acids -10 to 240 of NERD-L. In order to create the GST:NERD-L(S227A) mutant the pZd6 plasmid was mutagenized using the Oligonucleotide-directed *In vitro* Mutagenesis System (Amersham) with the following oligonucleotide:

5'-CCCGGAACGCGGCCTGAAT-3'.

This mutation destroys a StuI site in pZd6 (mutagenized nucleotide is underlined). The entire open reading frame was sequenced to insure polymerase fidelity in the mutagenesis reaction, and subcloned into pGEX-2T(Sac7) as above. Fusion proteins were produced and purified as described in Smith and Johnson (Smith and Johnson, 1988). Five micrograms of recombinant fusion protein was incubated for 10 minutes at 30°C with 30ng of purified rat PKC (Promega) in a 50µl reaction containing 20mM HEPES (pH 7.4), 0.4mM EDTA, 0.4mM EGTA, 1.7mM $CaCl_2$, 1mM DTT, 5µg phosphatidylserine, 0.5µg diolein, 250µM ATP and 1µl [γ-^{32}P]ATP (3000Ci/mmol). The reactions were stopped with SDS-loading buffer, boiled and 5µl were electrophoresed on a 12% SDS-PAGE gel, which was then fixed, dried and exposed to X-ray film for 4 hours at room temperature. Labeled bands were treated as in Wagner *et al.* (Wagner et al., 1991). Briefly, gel

slices corresponding to the fusion proteins were excised, soaked in 50% methanol/water for two hours and lyophilized. The labeled proteins were either treated for phosphoamino acid analysis or peptide mapping. For phosphoamino acid analysis, the dried samples were suspended in 5µl of pH 1.9 electrophoresis buffer (8% acetic acid: 2% formic acid) and placed in a glass screw cap tube with 200µl of 6N HCl. The tubes were evacuated with nitrogen gas and incubated in an oven at 110°C for 2 hours. The reactions were stopped by addition of 1ml of water and the hydrolyzed sample was dried by lyophilization. The pellet was resuspended in pH 1.9 buffer and spotted with phosphoamino acid standards on Kodak TLC cellulose plates. The plates were saturated with pH 1.9 buffer and electrophoresed at 500 volts until the phenol red indicator had migrated halfway across the plate. The plates were transferred to an electrophoresis chamber containing pH 3.5 buffer (10% acetic acid:1%pyridine) and electrophoresed until the indicator was 4cm from the end of the plate. After drying, the plates were sprayed with 1% ninhydrin to develop the standards and exposed at -70°C on X-ray film.

For 2-D proteolytic maps, the eluted and dried sample was digested overnight with thermolysin (100µg/ml) in 25mM ammonium bicarbonate. Lyophilized samples were resuspended in pH 1.9 buffer and spotted onto TLC plates . The plates were electrophoresed at 400V until the loading dye had migrated approximately 5 cm from the origin. The plates were dried and chromatographed in TLC buffer (butanol:pyridine:water:acetic acid,20:30:24:6) in a sealed glass chamber. After completion, the plates were driedand exposed to X-ray film at -70°C for 4 days.

Yeast Manipulations

Isolation of NERD-interacting proteins was based on the method of Gyuris and Brent (Gyuris and Brent, submitted) as outlined in Zervos *et al.* (Zervos et al., 1993). pEG202 was the backbone for constructing various LexA "baits". For the initial screen, an EcoRI/XhoI fragment from pZd10 coding for amino acids 159-240 of NERD-L was cloned into pEG202. This bait was cotransformed with pSH18-34 (a lacZ reporter plasmid) and a *Drosophila* 0-12 hour embryonic library (generously provided by Russ Finley) into the yeast strain EGY48 (*alpha, his3, trp1, ura3-52,* lex(*leu2*)3a). Transformed yeast were directly spread onto synthetic 2% galactose /1% raffinose (SGR) his^- ura^- trp^- leu^- plates supplemented with 50mM KH_2PO_4,(pH 7.0) and 40μg/ml X-gal to detect interacting proteins. Only blue, lacZ-expressing colonies were isolated. These were tested for galactose-dependent leu^+ activity by "patching" colonies to SD his^- ura^- trp^- leu^-. From approximately $4x10^6$ yeast colonies screened with NERD, 300 positive clones were isolated, and 12 were cloned. All these were found to carry portions of fly calmodulin (CaM). One of the CaM clones (pLBN6.15) was chosen for the remaining studies. pLBN6.15 is an in-frame fusion of *Drosophila* CaM that begins with amino acid 12 (Yamanaka et al., 1987) and extends to the C-terminus. To delineate the CaM binding domain(s) of NERD, various fragments of NERD-L were cloned into pEG202 as follows: The fusion in FigureIV.7 beginning with amino acid 41 was constructed by cutting pZd6 with NheI and HindIII. Klenow polymerase was used to blunt the ends and EcoR1 methylase was added to protect the internal EcoR1 site. EcoRI linkers were then ligated onto the fragment with T4 ligase, and EcoRI was added to generate sticky ends. The resulting fragment was gel-purified and ligated into an EcoRI-digested/alkaline phosphatase-treated pEG202 vector. In-frame fusions were confirmed by sequencing. For fusions beginning with amino acid 115 , pZd10 was digested

with DraIII, and the DNA was blunt-ended with T4 polymerase, and subsequently digested with XhoI. The vector for this insert was prepared by cutting pEG202 with BamHI, filling-in with Klenow polymerase, and digesting with XhoI. The constructs with mutant GAP43 modules were prepared as above using the Amersham kit and the following oligonucleotides:

T177E:5'-ATGACCACGGAATTCGGACTGAAT-3'

S227D:5'-GTGGCCCCGAAAGTCGGCCTGAAT-3'

T177E creates a novel EcoRI site and S227D eliminates a StuI site (mutagenized nucleotides are underlined). The double-mutant was prepared using the two oligonucleotides simultaneously in the mutagenesis reaction. Yeast transformation was carried out according to Gietz *et al.* (Gietz et al., 1992) with the modification of Hill *et al.* (Hill et al., 1991). LacZ measurements were made using a permeabilized cell assay based upon Guarente (Guarente, 1983) except that 1mM chlorophenol red-ß-D-galactopyranoside (Boehringer Mannhein) was substituted for *o*-nitrophenol-ß-D-galactoside and the reactions were carried out at room temperature and quenched after one minute with chilled 1M Na_2CO_3. ß-galactosidase activity units were defined as $OD_{570}/OD_{600}x1000$. Typically, background was +/-20 units. Experiments were done in duplicate and averaged. For any one construct these measurements did not vary by more than 20%.

Myristoylation Assay

In vitro RNA's were synthesized with T7 or SP6 polymerase and used in *in vitro* rabbit reticulocyte reactions (30µl) according to the manufacturer's specifications (Promega). 40µCi of ^{3}H-myristic acid or ^{35}S-methionine (Amersham) were added in labeling reactions as described in Li (Li and Aderem, 1992). The reactions were run on a 12% SDS-PAGE gel, fixed, immersed in 1M hydroxylamine (pH

7.0) overnight, soaked in Amplify (Amersham), dried on a slab gel-dryer, and exposed for 2-3 weeks at -70°C.

REFERENCES

Abeliovich, A., Chen, C., Goda, Y., Silva, A. J., Stevens, C. F. and Tonegawa, S. (1993a). Modified hippocampal long-term potentiation in PKC-gamma-mutant mice. Cell. 75, 1253-1262.

Abeliovich, A., Paylor, R., Chen, C., Kim, J. J., Wehner, J. M. and Tonegawa, S. (1993b). PKC-gamma mutant mice exhibit mild deficits in spatial and contextual learning. Cell. 75, 1263-1271.

Aderem, A. A., Albert, K. A., Keum, M. M., J.K., W., Greengard, P. and Cohn, Z. A. (1988). Stimulus-dependent myristoylation of a major substrate for protein kinase C. Nature. 332, 362-364.

Ahlijanian, M. K., Striessnig, J. and Catterall, W. A. (1991). Phosphorylation of an alpha1-like subunit of a omega-conotoxin-sensitive brain calcium channel by cAMP-dependent protein kinase and protein kinase C. J.Biol.Chem. 266, 20192-20197.

Aigner, L. and Caroni, P. (1993). Depletion of 43-kD growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. Journal of Cell Biology. 123, 417-429.

Alexander, K. A., Cimler, B. A., Meier, K. E. and Storm, D. R. (1987). Regulation of calmodulin binding to P-57. J. Biol. Chem. 262, 6108-6113.

Alexander, K. A., Wakim, B. T., Doyle, G. S., Walsh, K. A. and Storm, D. R. (1988). Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein. J. Biol. Chem. 263, 7545-7549.

Aloyo, V., Zwiers, H. and Gispen, W. H. (1983). Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 protein kinase. Journal of Neurochemistry. 41, 649-653.

Alphey, L., Jimenez, J., White-Cooper, H., Dawson, I., Nurse, P. and Glover, D. M. (1992). *twine*, a cdc25 Homolog That Functions in the Male and Female Germline of *Drosophila*. Cell. 69, 977-988.

Andersen, P. S., Smith, J. M. and Mygind, B. (1992). Characterization of the *upp* gene encoding uracil phosphoribosyltransferase of *Escherichia coli* K12. Eur.J.Biochem. 204, 51-56.

Andreasen, T. J., Luetje, C. W., Heideman, W. and Storm, D. R. (1983). Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. Biochemistry. 22, 4615-4618.

Ashburner, M. (1989). *Drosophila*: A Laboratory Manual. (Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press).

Au, D. C., Apel, E. D., Chapman, E. R., Estep, R. P., Nicolson, T. A. and Storm, D. R. (1989). Expression of cDNAs encoding wild-type and mutant neuromodulin in

Escherichia coli: comparision with the native protein from bovine brain. Biochemistry. 28, 8142-8148.

Baetge, E. E. and Hammang, J. P. (1991). Neurite outgrowth in PC12 cells deficient in GAP-43. Neuron. 6, 21-30.

Banasik, M., Komura, H., Shimoyama, M. and Ueda, K. (1992). Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribose) transferase. J. Biol. Chem. 267, 1569-1575.

Barrett, J. A. (1980). The estimation of the number of mutationally silent loci in saturation mapping experiments. Genetical Research. 35, 33-44.

Basi, G. S., Jacobson, R. D., Virag, I., Schilling, J. and Pate Skene, J. H. (1987). Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. Cell. 49, 785-791.

Baudier, J., Deloulme, J. C., Van Dorsselaer, A., Black, D. and Matthes, H. W. D. (1991). Purification and characterization of a brain-specific protien kinase C substrate, neurogranin(p17). J. Biol. Chem. 266, 229-237.

Baux, G. and Tauc, L. (1991). G proteins are involved in the regulation of transmitter release at an *Aplysia* cholinergic synapse. Brain Research. 575, 155-158.

Bazzi, M. D. and Nelsestuen, G. L. (1987). Role of Substrate in Imparting Calcium and Phospholipid Requirements to Protein Kinase C Activation. Biochemistry. 26, 1975-1982.

Beech, D. J., Bernheim, L. and Hille, B. (1992). Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. Neuron. 8, 97-106.

Belford, G. R. and Killackey, H. P. (1980). The sensitive period in the development of the trigeminal system of the neonatal rat. J.Comp.Neurol. 193, 335-350.

Ben-Ari, Y., Aniksztejn, L. and Bregestovski, P. (1992). Protein kinase C modulation of NMDA currents: an important link for LTP induction. TINS. 15, 333-339.

Benowitz, L. I., Perrone-Bizzozero, N. I. and Finklestein, S. P. (1987). Molecular properties of growth-associated protein GAP-43(B-50). Journal of Neurochemistry. 48, 1640-1647.

Benowitz, L. I., Rodriguez, W. R., Prusky, G. T. and Cynader, M. S. (1989). GAP-43 levels in cat striate cortex peak during the critical period. Neuroscience Abstracts. 15, 796.

Benowitz, L. I., Shashoua, V. E. and Yoon, M. G. (1981). Specific changes in rapidly-transported proteins during regenration of the golfish optic nerve. Journal of Neuroscience. 1, 300-307.

Bixby, J. L. (1989). Protein kinase C is involved in laminin stimulation of neurite outgrowth. Neuron. 3, 287-297.

Bliss, T. V. P. and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature361.

Bliss, T. V. P. and Lomo, T. (1973). J.Physiol. 232, 331-356.

Boland, L. and Bean, B. P. (1993). Inhibition of N-type calcium channels in bullfrog sympathetic neurons by leutenizing hormone releasing hormone. J.Neurosci. 13, 516-533.

Brady, S. T., Tyrell, M., Heriot, K. and Lasek, R. J. (1981). Axonal transport of calmodulin: A physiologic approach to identification of long-term associations between proteins. Journal of Cell Biology. 89, 607-614.

Bridges, P. N. (1935). J. Hered. 26, 60-64.

Buchsbaum, R., Buchsbaum, M., Pearse, J. and Pearse, V. (1987). Animals Without Backbones. (Chicago, University of Chicago Press).

Campos-Ortega, J. A. and Hartenstein, V. (1985). The embryonic development of *Drosophila melanogaster*. (New York, Springer-Verlag).

Carlin, R. K. and Siekevitz, P. (1983). Plasticity in the central nervous system: do synapse divide? Proc.Natl.Acad.Sci.USA. 80, 3517-3521.

Carr, S. A., Biemann, K., Shoji, S., Parmelee, D. C. and Titani, K. (1982). n-Tetradecanoyl is the amino-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. Proc.Natl.Acad.Sci. USA. 79, 6128-6131.

Cavener, D. R. (1987). Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. Nucleic Acids Res. 15, 1353-1361.

Chapman, E. R., Alexander, K., Vorherr, T., Carafoli, E. and Storm, D. R. (1992). Fluorescence Energy Transfer Analysis of Calmodulin-Peptide Complexes. Biochemistry. 31, 12819-12825.

Chapman, E. R., Au, D., Alexander, K. A., Nicolson, T. A. and Storm, D. R. (1991). Characterization of the calmodulin-binding domain of neuromodulin. J. Biol. Chem. 266, 207-213.

Chapman, E. R., Estep, R. P. and Storm, D. R. (1992). Palmitoylation of neuromodulin (GAP-43) is not required for phosphorylation by protein kinase C. J. Biol. Chem. 267, 25233-25238.

Cheney, R. E. and Mooseker, M. S. (1992). Unconventional Myosins. Current Opinion in Cell Biology. 4, 27-35.

Cimler, B. M., Giebelhaus, D. H., Wakin, B. T., Storm, D. R. and Moon, R. T. (1987). Characterization of murine cDNAs encoding P-57, a neural-specific calmodulin-binding protein. J. Biol. Chem. 262, 12158-12163.

Coggins, P. J., McLean, K., Nagy, A. and Zweirs, H. (1993a). ADP-ribosylation of neuronal phosphoprotein B-50/GAP-43. J.Neurochem. 60, 367-371.

Coggins, P. J., McLean, K. and Zwiers, H. (1993b). Neurogranin, a B-50/GAP-43-immunoreactive C-kinase substrate (BICKS), is ADP-ribosylated. FEBS Letters. 335, 109-113.

Cohen, R., Margulies, J. E., Coulter II, P. M. and Watson, J. B. (1993). Functional consequences of expression of the neuron-specific, protein kinase C substrate RC3 (neurogranin) in *Xenopus* oocytes. Brain Research. 627, 147-152.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V. A., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the *Drosophila* neurogenic genes in mesoderm differentiation. Cell. 67, 311-323.

Cormier, R. J., Mauk, M. D. and Kelly, P. T. (1993). Glutamate iontophoresis induce long-term potentiation in the absence of evoked presynaptic activityt. Neuron. 10, 907-919.

Costello, B., Norden, J. J. and Freeman, J. A. (1987). Induction and localization of the growth-associated protein, GAP-43, in NGF-stimulated PC12 cells. Soc.Neurosci.Abstr. 13,

Davis, M. B. and MacIntyre, R. J. (1988). A Genetic Analysis of the alpha-glycerophosphate Oxidase Locus in *Drosophila melanogaster*. Genetics. 120, 755-766.

Dekker, L. V., De Graan, P. N. E., Oestricher, A. B., Versteeg, D. H. G. and Gispen, W. H. (1989a). Inhibition of noradrenaline release by antibodies to B-50 (GAP-43). Nature. 342, 74-76.

Dekker, L. V., De Graan, P. N. E., Versteeg, D. H. G., Oestreicher, A. B. and Gispen, W. H. (1989b). Phosphorylation of B-50(GAP-43) is correlated with neurotransmitter release in rat hippocampal slices. Journal of Neurochemistry. 52, 24-30.

Deloulme, J.-C., Janet, T., Au, D., Storm, D. R., Sensenbrenner, M. and Baudier, J. (1990). Neuromodulin(GAP-43): a neuronal protein kinase C substrate is also present in O-2A glial cell lineage. Characterization of neuromodulin in secondary cultures of oligodendrocytes and comparison with the neuronal antigen. Journal of Cell Biology. 111, 1559-1569.

Delozanne, A. and Spudich, J. A. (1987). Science. 236, 1086-1091.

Dent, E. W. and Meiri, K. F. (1992). GAP-43 phosphorylation is dynamically regulated in individual growth cones. Journal of Neurobiology. 23, 1037-1053.

Di Luca, M., Merazzi, F., De Graan, P. N. E., Cimino, M., Balduini, W., Gispen, W. H. and Cattabeni, F. (1993). Selective alteration in B-50/GAP-43 phosphorylation

in brain areas of animals characterized by cognitive impairment. Brain Research. 607, 329-332.

DiAntonio, A., Burgess, R. W., Chin, A. C., Deitcher, D. L., Scheller, R. H. and Schwarz, T. L. (1993a). Identification and characterization of *Drosophila* genes for synaptic vesicle proteins. Journal of Neuroscience. 13, 4924-4935.

DiAntonio, A., Parfitt, K. D. and Schwarz, T. L. (1993b). Synaptic Transmission Persists in *synaptotagmin* Mutants of Drosophila. Cell. 73, 1281-1290.

Dorit, R., Schoenbach, L., Gilbert, W., and 7, (. 1. D. (1990). How big is the universe of exons? Science. 250, 1377-82.

Doster, S. K., Lozano, A. M., Aguayo, A. J. and Willard, M. B. (1991). Expression of the growth -associated protein GAP-43 in adult rat retinal ganglion cells following axonal injury. Neuron. 6, 635-647.

Duman, R. S., Terwilliger, R. Z. and Nestler, E. J. (1991). Endogenous ADP-ribosylation in brain: initial characterization of substrate proteins. Journal of Neurochemistry. 57, 2124-2134.

Duman, R. S., Terwilliger, R. Z. and Nestler, E. J. (1993). Alterations in nitric oxide-stimulated endogenous ADP-ribosylation associated with long-term potentiation in rat hippocampus. Journal of Neurochemistry. 61, 1542-1545.

Dumuis, A., Sebben, M., Haynes, L., Pin, J. P. and Bockaert, J. (1988). NMDA receptors activate the arachidonic acid cascade in striate neurons. Nature. 336, 68-70.

Eberl, D. F., Perkins, L. A., Engelstein, M., Hilliker, A. J. and Perrimon, N. (1992). Genetic and developmental analysis of polytene section 17 of the X chromosome of *Drosphila melanogaster*. Genetics. 130, 569-583.

Ehrlich, Y. H. and Routtenberg, A. (1974). Cyclic AMP regulates phosphorylation of three protein components of rat cerebral cortex membranes for thirty minutes. FEBS Letters. 45, 237-243.

Erzurumlu, R. S., Jhaveri, S. and Benowitz, L. I. (1990). Transient patterns of GAP-43 expression during the formation of barrels in the rat somatosensory cortex. J.Comp.Neurol. 292, 443-456.

Espreafico, E. M., Cheney, R. E., Matteoli, M., Nascimento, A. C., DeCamilli, P. V., Larson, R. E. and Mooseker, M. S. (1991). Journal of Cell Biology [abstract]. 115, 332a.

Fazeli, M. S. (1992). Synaptic plasticity: on the trail of the retrograde messenger. TINS. 15, 115-117.

Ferreira, A., Kincaid, R. and Kosik, K. S. (1993). Calcineurin is associated with the cytoskeleton of cultured neurons and ahs a role in the acquisition of polarity. Molecular Biology of the Cell. 4, 1225-1238.

Fields, S. (1993). The Two-Hybrid System to Detect Protein-Protein Interactions. Methods in Enzymology. 5, 116-124.

Fitzgerald, M., Reynolds, M. L. and Benowitz, L. I. (1991). GAP-43 expression in the developing rat lumbar spinal cord. Neurosience. 41, 187-199.

Fong, Y., Taylor, W. L., Means, A. R. and Soderling, T. R. (1989). J. Biol. Chem. 264, 16759-16763.

Forscher, P., Oxford, G. S. and Schulz, D. (1986). Noradrenaline modulates calcium channels in avian dorsal root ganglion cells through tight receptor-channel opening. J. Physiol. 379, 131-144.

Fujisawa, H., Tani, N., Watanabe, K. and Ibata, Y. (1982). Branching of regeneratig retinal axons and preferential selection of appropriate branches for specific neuronal connections in the newt. Developmental Biology. 90, 43-57.

Fujita, Y., Mynlieff, M., Dirksen, R. T., Kim, M., Niidome, T., Nakai, J., Friedrich, T., Iwabe, N., Miyata, T., Furuichi, T., Furutama, D., Mikoshiba, K., Mori, Y. and Beam, K. (1993). Primary structure and functional expression of the omega-conotoxin-sensitive N-type calcium channel from rabbit brain. Neuron. 10, 585-598.

Ghysen, A., Dambly-Chaudiere, C., Aceves, E., Jan, L.-Y. and Jan, Y.-N. (1986). Sensory neurons and peripheral pathways in *Drosophila* embryos. Roux's Arch Dev Biol. 195, 281-289.

Gianotti, C., Nunzi, M. G., Gispen, W. H. and Corradetti, R. (1992). Phosphorylation of the presynaptic protein B-50(GAP-43) is increased during electrically induced long-term potentiation. Neuron. 8, 843-848.

Gietz, D., St. Jean, A., Woods, R. A. and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20, 1425.

Gispen, W. H., Leunissen, J. L. M., Oestreicher, A. B., Verkleij, A. J. and Zwiers, H. (1985). Presynatic localization of B-50 phosphorylation: the ACTH-sensitive protein kinase substrate involved in rat brain polyphosphoinositide metabolism. Brain Research. 328, 381-385.

Gnegy, M. E., Nathanson, J. A. and Uzunov, P. (1977). Biochim.Biophys.Acta. 497, 75-85.

Graff, J. M., Gordon, J. I. and Blackshear, P. J. (1989). Myristoylated and non-myristoylated forms of a protein are phosphorylated by protein kinase C. Science. 246, 503-506.

Grassi, F. and Lux, H. D. (1989). Voltage-dependent GABA-induced modulation of calcium currents in chick sensory neurons. Neuroscience Letters. 105, 113-119.

Greene, L. A. and Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc.Natl.Acad.Sci.USA. 73, 2424-2428.

Guarente, L. (1983). Yeast promoters and *lacZ* fusions designed to study expression of cloend genes in yeast. Methods in Enzymology. 101, 181-191.

Halachmi,D. and Eilam,Y. (1993). Calcium homeostasis in yeast cells exposed to high concentrations of calcium. Roles of vacuolar H(+)-ATPase and cellular ATP. FEBS Lett. 316, 73-78.

Hartenstein, V. (1988). Development of *Drosophila* larval sensory organs: spatiotemporal pattern of sensory neurones, peripheral axonal pathways and sensilla differentiation. Development. 102, 869-886.

Hens, J. J. H., Benfenati, F., Nielander, H. B., Valtorta, F., Gispen, W. H. and De Graan, P. N. E. (1993a). B-50/GAP-43 Binds to Actin Filaments Without Affecting Actin Polymerization and Filament Organization. Journal of Neurochemistry. 61, 1530-1533.

Hens, J. J. H., De Wit, M., Dekker, L. V., Boomsma, F., Oestreicher, A. B., Margolis, F., Gispen, W. H. and De Graan, P. N. E. (1993b). Studies on the Role of B-50 (GAP-43) in the Mechanism of Ca+2-Induced Noradrenaline Release: Lack of Involvement of Protein Kinase C after the Ca+2 Trigger. Journal of Neurochemistry. 60, 1264-1273.

Hill, J., Ian, K. A., Donald, G. and D.E., G. (1991). DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res. 19, 5791.

Houbre, D., Duportail, G., Deloulme, J.-C. and Baudier, J. (1991). The interaction of the brain-specific calmodulin-binding protein kinase C substrate,

neuromodulin(GAP-43), with membrane phospholipids. J. Biol. Chem. 266, 7121-7131.

Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D. and Gilman, A. G. (1984). Science. 226, 860-862.

Ivins, K. J., Neve, K. A., Feller, D. J., Fidel, S. A. and Neve, R. L. (1993). Antisense GAP-43 inhibits the evoked release of dopamine from PC12. Journal of Neurochemistry. 60, 626-633.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. Proc.Natl.Acad.Sci.USA. 79, 2700-2704.

Johnston, G. C., Prendergast, J. A. and Singer, R. A. (1991). Journal of Cell Biology. 113, 539-551.

Jolles, J., Zwiers, H., van Dongen, C. J., Schotman, P., Wirtz, K. W. A. and Gispen, W. H. (1980). Modulation of brain polyphosphoinositide metabolism by ACTH-sensitive protein phosphorylation. Nature. 286, 623-625.

Karess, R. E. and Rubin, G. M. (1984). Analysis of P transposable element functions in *Drosophila*. Cell. 38, 135-146.

Karns, L. R., Ng, S.-C., Freeman, J. A. and Fishman, M. C. (1987). Cloning of cDNA for GAP-43, a neuronal growth-related protein. Science. 236, 597-600.

Kasai, H. (1991). Tonic inhibition and rebound facilitation of a neuronal calcium channel by a GTP-binding protein. Proc.Natl.Acad.Sci.USA. 88, 8855-8859.

Kasai, H. and Aosaki, T. (1989). Modulation of calcium channel currents by an adenosine analog mediated by a GTP-binding protein in chick sensory neurons. Pflugers Arch. 414, 145-149.

Katz, F., Ellis, L. and Pfenninger, K. H. (1985). Nerve growth cones isolated from fetal brain. Journal of Neuroscience. 5, 1402-1411.

Kenigsberg, R. L. and Trifaro, J. M. (1985). Micro-injection of calmodulin antibodies into cultured chromaffin cells blocks catecholamine release in response to stimulation. Neuroscience. 14, 335-347.

Kennelly, P. J. and Krebs, E. G. (1991). Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J. Biol. Chem. 266, 15555-15558.

Kern, L., de Montigny, J., Jund, R. and Lacroute, F. (1990). The *FUR1* gene of *Saccharomyces cerevisiae*: cloning, structure and expression of wild-type and mutant alleles. Gene. 88, 149-157.

Kidd, S. (1992). Characterization of the Drosophila *cactus* Locus and Analysis of Interactions between cactus and dorsal Proteins. Cell. 71, 623-635.

Kikkawa, U. and Nishizuka, Y. (1986). The role of protein kinase C in transmembrane signaling. Ann.Rev. Cell Biol. 2, 149-178.

Klambt, C., Jacobs, J. R. and Goodman, C. (1991). The Midline of the Drosophila Central Nervous System: A Model for the Genetic Analysis of Cell Fate, Cell Migration, and Growth Cone Guidance. Cell. 64, 801-815.

Klann, E., Chen, S.-J. and Sweatt, J. D. (1992). Increased phosphorylation of a 17-kDa protein kinase C substrate(p17/neurogranin) in long-term potentiation. Journal of Neurochemistry. 58, 1576-1579.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G. and Wittig, B. (1991). Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature. 353,

Knoll, A. (1992). The early evolution of eukaryotes: a geological perspective. Science. 256, 622-627.

Knyihar-Csillik, E., Csillik, B. and Oestreicher, A. B. (1992). Light and electron microscopic localization of B-50(GAP-43) in the rat spinal cord during transganglionic degenerative atrophy and regeneration. Journal of Neuroscience Research. 32, 93-109.

Kuo, C. and Bean, B. P. (1993). G-protein modulation of ion permeation through N-type calcium channels. Nature. 365, 258-262.

LaBate, M. E. and Pate Skene, J. H. (1989). Selective conservation of GAP-43 structure in vertebrate evolution. Neuron. 3, 299-310.

Leahy, J. C., Luo, Y., Kent, C. S., Meiri, K. F. and Vallano, M. I. (1993). Demonstration of presynaptic protein kinase C activation following long-term potentiation in rat hippocampal slices. Neuroscience. 52, 563-574.

Lee, K. S., Schottler, F., Oliver, M. and Lynch, G. (1980). Brief bursts of high-frequency stimulation produce structural change in rat hippocampus. J.Physiol. 44,

Li, J. and Aderem, A. (1992). MacMARCKS, A Novel Member of the MARCKS Family of Protein Kinase C Substrates. Cell. 70, 791-801.

Lin, L.-H., Bock, S., Carpenter, K., Rose, M. and Norden, J. J. (1992). Synthesis and transport of GAP-43 in entorhinal cortex neurons and perforant pathway during lesion-induced sprouting and reactive synaptogenesis. Molecular Brain Research. 14, 147-153.

Liu, Y., R., C. E. and Storm, D. R. (1991). Targeting of neuromodulin(GAP-43) fusion proteins to growth cones in cultured rat embryonic neurons. Neuron. 6, 411-420.

Liu, Y. and Storm, D. R. (1990). Regulation of free calmodulin: neuron growth and regeneration. TIPS. 11, 107-111.

Llinas, R., Sugimori, M. and Silver, R. B. (1992). Microdomains of high calcium concentration in a presynpatic terminal. Science. 256, 677-679.

Malenka, R. C., Madison, D. V. and Nicoll, R. A. (1986). Potentiation of synaptic transmission in the hippocampus by phorbol esters. Nature. 321, 175-177.

Malenka, R. C. and Nicoll, R. A. (1993). NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms. TINS. 16, 521-527.

Malinow, R., Madison, D. V. and Tsien, R. W. (1988). Persistent protein kinase activity underlying long-term potentiation. Nature. 335, 820-824.

Maness, P. F., Aubry, M., Shores, C. G., Frame, L. and Pfenninger, K. H. (1988). The c-src gene product in developing rat brain is enriched in nerve growth cone membranes. Proc. Natl. Acad. Sci. 85, 5001-5005.

Masure, H. R., Alexander, K. A., B.T., W. and Storm, D. R. (1986a). Physicochemical and hydrodynamic characterization of P-57, a neurospecific calmodulin binding protein. Biochemistry. 25, 7553-7560.

Masure, H. R., Alexander, K. A., B.T., W. and Storm, D. R. (1986b). Physicochemical and hydrodynamic characterization of P-57, a neurospecific calmodulin binding protein. Biochemistry. 25, 7553-7560.

Matthies, H. J., Palfrey, H. C. and Miller, R. J. (1988). Calmodulin- and protein phosphorylation-independent release of catecholamines in PC12 cells. FEBS Letters. 229, 238-242.

McEnery, M. W., Snowman, A. M. and Snyder, S. H. (1994). The association of endogenous Go with the purified omega-conotoxin GVIA receptor. J. Biol. Chem. 269, 5-8.

Meiri, K. and Gordon-Weeks, P. R. (1990). GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. Journal of Neuroscience. 10, 256-266.

Meiri, K. F., Bickerstaff, L. E. and Schwob, J. E. (1991). Monoclonal antibodies show that Kinase C phosphorylation of GAP-43 during axonogenesis is both spatially and temporally resticted *in vivo*. Journal of Cell Biology. 112, 991-1005.

Meiri, K. F., Pfenninger, K. H. and Willard, M. B. (1986). Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. Proc.Natl.Acad.Sci.USA. 83, 3537-3541.

Mercer, J. A., Seperack, P. K., Copeland, N. G. and Jenkins, N. A. (1991). Nature. 349, 709-712.

Mills, L. R. and Kater, S. B. (1990). Neuron-specific and state-specific differences in calcium homeostasis regulate the generation and degeneration of neuronal architecture. Neuron. 2, 149-163.

Montell, C. and Rubin, G. M. (1988). The Drosophila *ninaC* locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. Cell. 52, 757-772.

Moss, D. J., Fernyhough, P., Chapman, K., Baizer, L., Bray, D. and Allsopp, T. (1990). Chicken growth-associated protein GAP-43 is tightly bound to the actin-rich neuronal membrane skeleton. Journal of Neurochemistry. 54, 729-736.

Moss, J. and Vaughan, M. (1978). Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase. Proc.Natl.Acad.Sci.USA. 75, 3621-3624.

Murzin, A. G. (1993). Can homologous proteins evolve different enzymatic activities. Trends in Biological Sciences. 18, 403-405.

Neve, R. L., Finch, E. A., Bird, E. D. and Benowitz, L. I. (1988). Growth-associated protein GAP-43 is expressed selectively in associative regions of the adult human brain. Proc. Natl.Acad.Sci. USA. 85, 3638-3642.

Ng, S.-C., Perkins, L. A., Conboy, G., Perrimon, N. and Fishman, M. C. (1989). A *Drosophila* gene expressed in the embryoninc CNS shares one conserved domain with mammalian GAP-43. Development. 105, 629-638.

Nielander, H. B., Schrama, L. H., Van Rozen, A. J., Kasperaitis, M., Oestreicher, A. B., Gispen, W. H. and Schotman, P. (1990). Mutation of Serine 41 in the Neuron-specific Protein B-50(GAP-43) Prohibits Phosphorylation by Protein Kinase C. Journal of Neurochemistry. 55, 1442-1445.

Nishizuka, Y. (1986). Studies and Perspectives of Prpotein Kinase C. Science. 232, 305-312.

O'Dell, T. J., Hawkins, R. D., Kandel, E. R. and Arancio, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. Proc.Natl.Acad.Sci.USA. 88, 11285-11289.

Pearson, W. R. and Lipman, D. J. (1988). Improved tools for biological comparison. Proc.Natl.Acad.Sci.USA. 85,

Perkel, D. J., Petrozzino, J. J., Nicoll, R. A. and Connor, J. A. (1993). The role of Ca+2 entry via synaptically activated NMDA receptors in the induction of long-term potentiation. Neuron. 11, 817-823.

Pirrotta, V., Steller, H. and Bozzetti, M. P. (1985). Multiple upstream regulatory elements control the expression of the *Drosophila white* gene. EMBO J. 4, 3501-3508.

Porter, J. A., Yu, M., Doberstein, S. K., Pollard, T. D. and Montell, C. (1993). Dependence of Calmodulin Localization in the Retina on the NINAC Unconventional Myosin. Science. 262, 1038-1042.

Rakic, P. (1981). Development of visual centers in the primate brain depends on binocular competition before birth. Science. 214, 928-931.

Represa, A., Deloulme, J. C., Sensenbrenner, M., Ben-Ari, Y. and Baudier, J. (1990). Neurogranin: immunocytochemical localization of a brain-sprecific protein kinase C substrate. Journal of Neuroscience. 10, 3782-3792.

Robinson, P. J. (1991). The role of protein kinase C and its neuronal substrates dephosphin, B-50, and MARCKS in neurotransmitter release. Molecular Neurobiology. 5, 87-130.

Rosenthal, A., Chan, S. Y., Henzel, W., Haskell, C., Kuang, W.-J., Chen, E., Wilcox, J. N., Ullrich, A., Goeddel, D. V. and Routtenberg, A. (1987). Primary structure and mRNA localization of protein F1, a growth-related protein kinase C substrate associated with synaptic plasticity. EMBO J. 6, 3641-3646.

Routtenberg, A. (1985). Protein kinase C activation leading to protein F1 phosphorylation may regulate synaptic plasticity by presynaptic terminal growth. Behavioral and Neural Biology. 44, 186-200.

Routtenberg, A., Lovinger, D. and Steward, O. (1985). Phosphorylation of a Kinase C substrate is directly related to LTP in the intact hippocampus. Behavioral and Neural Biology. 43, 3-11.

Rubin, G. M. and Spradling, A. C. (1983). Vectors for P-element mediated gene transfer in *Drosophila*. Mol.Cell.Biol. 11, 6341-6351.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, New York, Cold Spring Harbor Laboratory).

Sangameswaran, L., Hempstead, J. and Morgan, J. I. (1989). Molecular cloning of a neuron-specific transcript and its regulation during normal and aberrant cerebellar development. Porc. Natl.Acad.Sci. USA. 86, 5651-5655.

Schaechter, J. D. and Benowitz, L. I. (1993). Activation of protein pinase C by arachidonic acid selectively enhances the phosphorylation of GAP-43 in nerve terminal membranes. Journal of Neuroscience. 13, 4361-4371.

Scholz, K. P. and Miller, R. J. (1991). Analysis of adenosine actions on calcium currents and synaptic transmission in cultured hippocampal pyramidal neurones. Journal of Physiology. 435, 373-393.

Schultz, A. M., Henderson, L. E., Oroszlan, S., Garber, E. A. and Hanafusa, H. (1985). Amino terminal myristoylation pf the protein kinase p60src, a retroviral transforming protein. Science. 227, 427-429.

Schuman, E. M., Meffert, M. K., Schulman, H. and Madison, D. V. (1992). A potential role for an ADP-ribosyltransferase in hippocampal long-term potentiation (LTP). Soc.Neurosci.Abstr. 18, 761.

Sefton, B. M. and Buss, J. E. (1987). The covalent modification of eukaryotic protiens with lipid. Journal of Cell Biology. 104, 1449-1453.

Seykora, J. T., Ravetch, J. V. and Aderem, A. (1991). Cloning and molecular charachterization of the murine macrophage "68-kDa" protein kinase C substrate

and its regulation by bacterial lipopolysaccharide. Proc.Natl.Acad.Sci. USA. 88, 2505-2509.

Shea, T. B., Perrone-Bizzozero, N. I., Beermann, M. L. and Benowitz, L. I. (1991). Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neuritogenesis. Journal of Neuroscience. 11, 1685-1690.

Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. and Nishizuka, Y. (1991). Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinace C activation. Proc.Natl.Acad.Sci.USA. 88, 5149-5153.

Skene, J. H. P. (1981). Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. Journal of Cell Biology. 89, 86-95.

Skene, J. H. P. and Virag, I. (1989). Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. Journal of Cell Biology. 108, 613-624.

Skene, J. H. P. and Willard, M. (1981a). Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous system. Journal of Cell Biology. 89, 96-103.

Skene, J. H. P. and Willard, M. (1981b). Characteristics of growth-associated polypeptides in regenerating toad retinal ganglion cell axons. J.Neurosci. 1, 419-426.

Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-*S*-transferase. Gene. 67, 31-40.

Sobue, K. and Kanda, K. (1989). Alpha-actinins, calspectin (brain spectrin or fodrin), and actin participate in adhesion and movement of growth cones. Neuron. 3, 311-319.

Sommervaille, T., Reynolds, M. L. and Woolf, C. J. (1991). Time-dependent differences in the increase in GAP-43 expression in dorsal root ganglion cells after peripheral axotomy. Neuroscience. 45, 213-220.

Soong, T. W., Stea, A., Hodson, C. D., Dubel, S. J., Vincent, S. R. and Snutch, T. P. (1993). Structure and functional expression of a member of the low-voltage-activated calcium channel family. Science. 260, 1133-1136.

Spencer, S. and Willard, M. B. (1992). Does GAP-43 support axon growth by increasing the axonal transport velocity of calmodulin? Experimental Neurology. 115, 167-172.

Steele, F. R., Washburn, T., Rieger, R. and O'Tousa, J. E. (1992). Drosophila *retinal degeneration C* (*rdg*C) encodes a novel serine/threonine phosphatase. Cell. 69, 669-676.

Stocker, K. M., Baizer, L. and Ciment, G. (1992). Transient expression of GAP-43 in nonneuronal cells of the embryonic chicken limb. Developmental Biology. 149, 406-414.

Strittmatter, S. M., Cannon, S. C., Ross, E. M., Higashijima, T. and Fishman, M. C. (1993). GAP-43 augments G protein-coupled receptor transduction in *Xenopus laevis* oocytes. Proc.Natl.Acad.Sci.USA. 90, 5327-5331.

Strittmatter, S. M., Valenzuela, D., Kennedy, T. E., Neer, E. J. and Fishman, M. C. (1990). G_0 is a major growth cone protein subject to regulation by GAP-43. Nature. 344, 836-841.

Strittmatter, S. M., Vartanian, T. and Fishman, M. C. (1992). GAP-43 as a Repair Protein in Neuronal Form and Repair. Journal of Neurobiology. 23, 507-520.

Sudo, Y., Valenzuela, D., Beck-Sickinger, A. G., Fishman, M. C. and Strittmatter, S. M. (1992). Palmitoylation alters protein activity: blockade of G_0 stimulation by GAP-43. EMBO J. 11, 2095-2102.

Swanljung-Collins, H. and Collins, J. H. (1992). Phosphorylation of brush border myosin I by protein kinase C is regulated by calcium-stimulated binding of myosin I to phosphatidylserine concerted with calmodulin dissociation. J. Biol. Chem. 267, 3445-3454.

Swartz, K. J. and Bean, B. P. (1992). Inhibition of calcium channels in rat CA3 pyramidal cells neurons by a metabotropic glutamate receptor. J.Neurosci. 12, 4358-4371.

Swartz, K. J., Merritt, A., Bean, B. P. and Lovinger, D. M. (1993). Protein kinase C modulates glutamate receptor inhibition of Ca2+ channels and synaptic transmission. Nature. 361, 165-168.

Takagi, T. and Cox, J. A. (1990). Primary structure of the target of calcium vector protein(CaVP) of *Amphioxus*. J. Biol. Chem. 265, 19721-19727.

Taussig, R., Sanchez, S., Rifo, M. and Gilman, A. G. (1992). Inhibition of the omega-conotoxin-sensitive calcium current by distinct G-proteins. Neuron. 8, 799-809.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for localization of specific mRNAs in *Drosophila* embryos reveals translation control of the segmentation gene hunchback. Chromosoma. 98, 81-85.

Taylor, D., Sack, J. S., Maune, J. F., Beckingham, K. and Quiocho, F. A. (1991). Structure of a Recombinant Calmodulin from *Drosophila melanogaster* Refined at 2.2-Å Resolution. J. Biol. Chem. 266, 21375-21380.

Thelen, M., Rosen, A., Nairn, A. and Aderem, A. (1991). Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. Nature. 351, 320-322.

Towler, D. A., Gordon, J. I., Adams, S. P. and Glaser, L. (1988). The biology and enzymology of eukaryotic protein acylation. Ann.Rev.Biochem. 57, 69-99.

Ueda, K. and Hayaishi, O. (1985). ADP-ribosylation. Ann.Rev.Biochem. 54, 73-100.

Underwood, E. M., Briot, A. S., Doll, K. Z., Ludwiczak, R. L., Otteson, D. C., Tower, J., Vessey, K. B. and Yu, K. (1990). Genetics of 51D-52A, a region containing several maternal-effect genes and two maternal-specific transcripts in *Drosophila*. Genetics. 126, 639-650.

Van Dongen, C. J., Zwiers, H., De Graan, P. N. E. and Gispen, W. H. (1985). Modulation of the activity of purified phospatidylinositol 4-phosphate kinase by phosphorylated and dephosphorylated B-50 protein. Biochem.Biophys.Res.Comm. 128, 1219-1227.

Van Dop, C., Tsubokawa, M., Bourne, H. and Ramchandran, J. (1984). J. Biol. Chem. 259,

Wagner, K., Edson, K., Heginbotham, L., Post, M., Huganir, R. L. and Czernik, A. J. (1991). Determination of the tyrosine phosphorylation sites of the nicotinic acetylcholine receptor. J. Biol. Chem. 266, 23784-23789.

Wakim, B. T., Alexander, K. A., Masure, H. R., Cimler, B. M., Storm, D. R. and Walsh, K. A. (1987). Amino acid sequence of P-57, a neurospecific calmodulin-binding protein. Biochemistry. 26, 7466-7470.

Walters, E. T., Alizadeh, H. and Castro, G. A. (1991). Similar neuronal alteration induced by axonal injury and learning in *Aplysia*. Science. 253, 797-799.

Wang, J. K., Walaas, S. I., Sihra, T. S. and Aderem, A. A. (1989). Phosphorylation and associated translocation of the 87-kD protein, a major protein kinase C substrate, in isolated nerve terminals. Proc. Natl.Acad.Sci. USA. 86, 2253-2256.

Widmer, F. and Caroni, P. (1993). Phosphorylation-Site Mutagenesis of the Growth-Associated Protein GAP-43 Modulates Its Effects on Cell Spreading and Morphology. Journal of Cell Biology. 120, 503-512.

Wilk-Blaszczak, M. A., Gutowski, S., Sternweis, P. C. and Belardetti, F. (1994). Bradykinin modulates potassium and calcium currents in neuroblastoma hybrid cells via different pertussis toxin-insensitive pathways. Neuron. 12, 109-116.

Williams, E. J. D., P., Turner, G., Reid, R. A., Hemperly, J. J. and Walsh, F. S. (1992). Calcium influx into neurons can solely account for cell-contact-dependent neurite outgrowth stimulated by transfected L1. Journal of Cell Biology. 119, 883-892.

Yamanaka, M. K., Saugstad, J. A., Hanson-Painton, O., McCarthy, B. J. and Tobin, S. L. (1987). Structure and expression of the *Drosophila* calmodulin gene. Nucleic Acids Res. 15, 3335-3348.

Zervos, A. S., Gyuris, J. and Brent, R. (1993). Mxi1, a Protein That Specifically Interacts with Max to Bind Myc-Mac Recognition Sites. Cell. 72, 223-232.

Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschick, P. and Spiegel, A. M. (1986). Multisite phosphorylation of the alph subunit of transducin by the insulin receptor kinase and PKC. Proc.Natl.Acad.Sci.USA. 83, 9294-9297.

Zuber, M. X., Strittmatter, S. M. and Fishman, M. C. (1989). A membrane-targeting signal in the amino-terminus of the neuronal protein GAP-43. Nature. 341, 345-348.

Zwiers, H., Wiegant, V. M., Schotman, P. and Gispen, W. H. (1978). ACTH-induced inhibition of endogenous rat brain protein phosphorylation *in vitro*. Neurochem. Res. 3, 455-463.

End