# THE ROLE OF PROTEIN KINASE Cγ IN MAMMALIAN CENTRAL NERVOUS SYSTEM FUNCTION: A GENETIC APPROACH

By ASA ABELIOVICH

B.S. Biology, Massachusetts Institute of Technology, 1986
B.S. Humanities, Massachusetts Institute of Technology, 1986

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Signature of Author _____

Department of Biology
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Certified by _____

Susumu Tonegawa
Thesis Supervisor

Accepted by _____

Frank Solomon, Chairman
Biology Department Graduate Committee

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## ABSTRACT

Calcium-phospholipid-dependent protein kinase (PKC) has been implicated in both synaptic plasticity and learning. To investigate the role of PKC in mechanisms of synaptic plasticity, and to relate mechanisms of synaptic plasticity to learning, mice deficient in the γ isotype of PKC were generated. Mutant mice are viable, develop normally, and have synaptic transmission that is indistinguishable from wild-type mice. Long-term potentiation of synaptic transmission (LTP) is absent or greatly attenuated in PKCγ mutant mice, while two other forms of synaptic plasticity, long-term depression and paired-pulse facilitation, are normal. When tetanus to evoke LTP is preceded by a low-frequency stimulation, however, mutant animals display apparently normal LTP. This suggests that PKCγ is not part of the molecular machinery that produces LTP, but may play a role in regulating or modulating LTP.

To investigate the roles of PKCγ and synaptic plasticity mechanisms in learning and memory, PKCγ mutant mice were tested in two hippocampus-dependent tasks, the hidden-platform Morris water maze and context-dependent fear conditioning. PKCγ mutant mice were able to carry out both tasks, although mild deficits were evident. Thus, hippocampal LTP produced by the conventional tetanic stimulation is not essential for the mice to exhibit spatial and contextual learning. The data as a whole is most consistent with a role for multiple forms of synaptic plasticity, including LTP and LTD, in hippocampus-dependent learning and memory. Furthermore, the modulation of LTP may play an important role in hippocampus-dependent learning and memory.

Thesis Supervisor: Dr. Susumu Tonegawa
Title: Professor

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The really important facts were that spatial relationships had ceased to matter very much and that my mind was perceiving the world in terms of other than spatial categories. At ordinary times the eye concerns itself with such problems as *Where? – How far? -- How situated in relation to what?* In the mescaline experience the implied questions to which the eye responds are of another order. Place and distance ceased to be of much interest. The mind does its perceiving in terms of intensity of experience, profundity of significance, relationships within a pattern.

-- Aldous Huxley, *The Doors of Perception*

These fragments I have shored against my ruins
Why then Ile fit you.

-- T.S. Eliot , "The Waste Land"

# Table of Contents

# CHAPTER 1

# INTRODUCTION

# SIGNAL TRANSDUCTION IN THE MODULATION OF SYNAPTIC STRENGTH:

# THE ROLE OF PKC IN LTP

## SUMMARY

**Protein kinase C (PKC) appears to play a complex role in long-term potentiation of synaptic transmission (LTP), a form of synaptic modulation that has been implicated in mammalian learning and memory. Pharmacological studies indicate that PKC activation is necessary but not sufficient to induce LTP. Some reports suggest that PKC directly relays the calcium signal required for LTP induction, while others suggest that PKC modulates LTP induction. Additionally, a constituitively active form of PKC has been implicated in the expression of LTP. Different PKC isotypes may serve different functions in LTP. Analysis of genetically altered mice supports a role for the $\gamma$ isotype of PKC in the modulation of LTP.**

## INTRODUCTION

Long-term potentiation (LTP) of synaptic transmission in the mammalian hippocampus has been implicated as an important physiological mechanism that underlies certain forms of learning and memory. Initially, this was proposed on strictly theoretical grounds; Donald Hebb postulated that long-lasting, activity-dependent synaptic potentiation underlies learning and memory (Hebb, 1949). Subsequently, a form of synaptic potentiation was discovered that is robustly present in the hippocampus (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973), a CNS structure that has been shown to play an important role in certain types of learning through lesion experiments (see Eichenbaum et al., 1992; Hirsh, 1974; O'Keefe and Nadel, 1978; Jarrard, 1993). In agreement with

Hebb's postulate, pharmacological (Morris et al., 1981, 1982, 1986) and genetic (Silva et al., 1992a, 1992b; Grant et al., 1992; Abeliovich et al., 1993a, 1993b) manipulations that disrupt LTP or the regulation of LTP in the hippocampus have been found also to disrupt explicit, complex forms of learning -- forms of learning that are sensitive to lesions of the hippocampus formation. Thus, LTP has been implicated as a physiological substrate for learning and memory.

To gain insight into cellular mechanisms that underlie mammalian learning and memory, considerable attention has been focused on the molecular mechanism and regulation of LTP. Indeed, much is now known about the basic mechanism by which LTP is initiated, and certain key players have been identified (see Bliss and Collingridge, 1993). LTP is most commonly studied at synapses in the hippocampus between Schaffer collateral fibers, which arise from CA3 pyramidal cells, and dendrites of CA1 pyramidal cells. Synaptic transmission at these synapses is initiated by an action potential which travels down the axon (Schaffer collateral fibers) and depolarizes the presynaptic terminals. Activation of voltage-gated calcium channels at the presynaptic terminus leads to calcium influx, which in turn induces the fusion of a pool of synaptic vesicles to the presynaptic plasma membrane and the release of glutamate into the synaptic cleft. Glutamate receptors on the postsynaptic membrane, primarily of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) class, bind released glutamate and open intrinsic ion channels, resulting in an influx of sodium and depolarization of the postsynaptic membrane. This depolarization is typically measured either as a field potential (excitatory post-synaptic potential or EPSP) using extracellular recording electrodes, or as current flow into the cell at a fixed membrane potential (excitatory post-synaptic currents or EPSCs) using patch electrodes. The synaptic strength of this pathway is

typically monitored by stimulating Schaffer collateral axons with a current pulse and recording elicited responses in either CA1 cell bodies or in the CA1 dendritic field.

A series of high-frequency (100 Hz) bursts of stimulation to the Schaffer collateral pathway produces a robust increase in synaptic strength lasting an hour or longer, a phenomenon termed long-term potentiation (LTP). LTP in the CA1 region of the hippocampus displays so-called "Hebbian" properties: coincident presynaptic and postsynaptic activity is required for its induction (termed activity-dependence); the modulation is specific for the activated synapse; and it is long-lasting. High-frequency bursts of stimulation lead to massive input to and depolarization of both the presynaptic and the postsynaptic cells, and therefore serve to satisfy the activity-dependence of LTP. LTP also displays associativity and cooperativity, in that an input to a given postsynaptic cell will display LTP if it is active coincident with high-frequency stimulation of another pathway to the same postsynaptic cell, and in that multiple, coincidentally active inputs to the same postsynaptic cell can synergistically induce LTP. Another phenomenon with very similar properties to LTP, but involving a depression of synaptic transmission, has also been observed at Schaffer collateral synapses. Long term depression (LTD) of synaptic transmission can be induced by low-frequency stimulation (1 Hz) of this pathway (Dudek and Bear, 1992; Mulkey and Malenka, 1992).

The molecular basis of LTP induction has been partly elucidated. The activity dependence of LTP results from the unique properties of a glutamate receptor class, the N-methyl-D-aspartate (NMDA) receptor. NMDA receptors possess two unique characteristics. First, NMDA receptors require both

glutamate binding and depolarization of the postsynaptic cell (depolarization relieves a magnesium-dependent block of the channel) in order to be activated (Mayer et al., 1984; Nowack et al., 1984); therefore, NMDA receptors detect coincident activity in presynaptic and postsynaptic neurons. Second, NMDA receptors, upon activation, flux calcium as well as sodium through an intrinsic ion channel (Ascher and Nowack, 1988; Jahr and Stevens, 1987; Mayer et al., 1987). Calcium influx through NMDA receptors is likely to be the initial trigger for LTP, as either calcium chelators (Lynch et al., 1983; Malenka et al., 1988) or NMDA receptor channel blockers (Collingridge, 1983) can block the induction of LTP . However, calcium influx appears to be insufficient for LTP induction (Malenka, 1989b; Malenka et al., 1988); Grover and Teyler, 1990), and it is possible that other signals, such as metabotropic glutamate receptor activation (see below), are also required. Subsequent events in the induction and expression of LTP, and the regulation of these events, remain largely unknown. Research has focused largely on second messenger pathways activated by calcium influx through the NMDA receptor, and PKC has been a primary focus of attention.

Little is known about the locus of change that underlies LTP. Considerable debate has arisen over whether LTP is ultimately the consequence of increased release of glutamate from the presynaptic terminal or increased sensitivity to glutamate at the postsynaptic receptor. Indirect evidence in favor of a presynaptic change in the probability of glutamate release comes from the quantal analysis of synaptic transmission (see Stevens, 1993), although a postsynaptic change has also been invoked in some of these studies (Kullman and Nicoll, 1992; Larkman et al., 1992). Quantal analysis is an indirect approach based on the assumption that the probability of release of a given number of

transmitter packets in response to stimulation is strictly a presynaptic parameter, whereas the size of the response to a transmitter packet reflects postsynaptic receptor sensitivity. Therefore, changes in the probability of failed transmission (of zero neurotransmitter packets being released) at a given synapse would reflect changes in presynaptic function. Upon the induction of LTP, the probability of transmitter release does appear to be increased, consistent with a presynaptic mechanism underlying LTP. However, it is important to note that direct evidence for a presynaptic mechanism of LTP induction is lacking.

Since LTP appears to be induced by postsynaptic calcium influx through NMDA receptors, any consequent presynaptic changes invoke retrograde signaling from the postsynaptic to the presynaptic cell ( see Jessell and Kandel, 1993). Intercellular second messengers such as nitric oxide and carbon monoxide have been implicated as potential retrograde messengers in LTP. Inhibitors of the production of both nitric oxide (Bohme et al., 1991; O'Dell et al., 1991a; Schuman and Madison, 1991) and carbon monoxide (Zhou et al., 1993; Stevens and Wang, 1993) have been reported to block the induction of LTP, while production of NO or CO can stimulate LTP induction (Zhou et al., 1993). However, none of these studies directly address whether a retrograde messenger is involved in LTP.

**PKC in LTP**

Little is known about the precise signal transduction pathways that underlie LTP. Several kinases have been implicated, including calcium-calmodulin-dependent protein kinase (CaMKII), calcium-phospholipid-dependent protein kinase (PKC), and tyrosine kinases, and PKC has probably received the

most attention. Evidence of a role for PKC in LTP comes primarily from experiments that utilize PKC activators and inhibitors in order to either mimic or inhibit LTP. Some studies have investigated the role of PKC in the signaling mechanism of LTP. Other studies provide evidence that PKC may play a role in the regulation or modulation of LTP. Finally, a few studies have attempted to identify potential substrates of PKC.

**PKC Activators and LTP**. A number of studies have addressed the effects of phorbol esters, activators of PKC, on synaptic transmission. Phorbol esters are structural analogs of diacylglycerol (DAG), a potent activator of PKC generated in vivo by phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (as well as phospholipase D-mediated hydrolysis of phosphatidylcholine and other phospholipids; see Nishizuka, 1992). Although phorbol esters do potentiate synaptic transmission, the data as a whole suggests that phorbol ester-mediated potentiation of synaptic transmission is short-lived and differs mechanistically from LTP.

Initial reports suggested that brief application of phorbol esters alone induce a long-lasting potentiation of synaptic transmission that mimics LTP (Malenka et al., 1986). Consistent with this, intracellular injection of PKC (a mixture of isotypes) into the postsynaptic CA1 pyramidal cells has been reported to mimic LTP (Hu et al., 1987). Furthermore, upon LTP induction, PKC appears to be translocated to a membrane-associated compartment, suggesting that LTP induction involves PKC activation (Akers et al., 1986). These initial studies implicated PKC as a nodal point in the induction of LTP; PKC may relay the NMDA-receptor mediated calcium influx signal to unknown substrates (Fig. 1A). Consistent with the idea that PKC is intimately involved in signaling for LTP

induction, quantal analysis of phorbol ester-mediated synaptic potentiation (Yamamoto et al., 1986) and measurement of glutamate release from hippocampal slices treated with phorbol esters (Malenka et al., 1987) both suggest that phorbol ester-mediated potentiation is similar to LTP in that it is ultimately a consequence of an increase in glutamate release from the presynaptic terminal.

Several subsequent studies have challenged the notion that PKC activation is sufficient for the induction of LTP. Malinow et al. (1988) improved the phorbol ester washout technique and found that application of phorbol esters alone does not induce LTP, but rather elicits a decremental potentiation of synaptic transmission lasting less than an hour. This effect appears similar in time course to another phenomenon, short term potentiation (STP), which is observed when CA1 cells are given a weak high-frequency stimulation insufficient to elicit LTP. STP, like LTP, is dependent on calcium influx through the NMDA receptor, and it has been suggestedthat STP plays a significant physiological role (Colino et al., 1992). The induction requirements for STP must differ either quantitatively or qualitatively from those for LTP. It is possible that PKC activation is sufficient to induce STP, and that perhaps other signals, such as activation of CamKII (Malinow et al., 1989) or tyrosine kinases (O'Dell et al., 1991b), are required for LTP induction (Fig. 1B). Alternatively, PKC-mediated potentiation may be mechanistically different from LTP, or PKC may play separate roles in these two processes. In support of these latter models, previous LTP does not affect subsequent phorbol ester-mediated potentiation (Gustafsson et al., 1988; Muller et al., 1988). Application of phorbol esters can inhibit the induction of LTP under certain conditions, but this is most likely not due to occlusion (saturation of a common underlying mechanism, suggesting that this

mechanism is shared by the processes) but rather to inhibitory phorbol ester effects (such as channel modulation; see below), as occlusion should not depend on the order of induction of the two mechanisms. Additionally, Gustafsson et al. (1988) investigated the interaction between phorbol ester-mediated synaptic potentiation and paired-pulse facilitation (PPF). PPF is a short-lived form of synaptic enhancement (lasting several hundred milliseconds) in which the second of a pair of stimuli is strengthened relative to the first. Interestingly, previous phorbol ester-mediated potentiation appears to occlude PPF, whereas LTP does not (McNaughton, 1982). Thus, LTP and phorbol ester-mediated potentiation appear to be mechanistically different, although both forms of synaptic enhancement may ultimately involve an increase in glutamate release from the presynaptic terminal.

**PKC Inhibitors**. General protein kinase inhibitors and specific PKC inhibitors have been informative in elucidating the role of PKC in LTP. Initial studies took advantage of the availability of pharmacological agents that block either kinase activation or kinase activity per se. Additional studies have utilized more specific agents or direct intracellular application of an agent. These experiments suggest a requirement for PKC in LTP. However, these experiments are sometimes limited by the degree of specificity of the pharmacological agents used, as a number of PKC inhibitors (such as H-7) block kinases other than PKC. Additionally, it cannot be concluded from any of these experiments that PKC plays a role in the actual process of LTP induction, rather than in a requisite enabling process that takes place prior to LTP induction, because inhibitors are always applied before LTP induction in order to equilibrate the agent (see Perkel and Nicoll, 1991).

Sphingosine, a drug that blocks the activation of protein kinases, and H-7, a drug that blocks protein kinase activity, have both been reported to block the initiation of LTP (Malinow et al., 1988). The presence of either sphingosine or H-7 during high-frequency stimulation prevents LTP induction and results in a decremental potentiation that mimics STP. Intracellular injection of H-7 into the postsynaptic CA1 cell also appears to prevent the initiation of LTP (Malinow et al., 1989). Additional studies utilizing drugs with improved specificity have confirmed and extended these results (Malinow et al., 1989). Intracellular injection of the peptide pseudosubstrate PKCI (19-31), a highly specific inhibitor of PKC activity, into the postsynaptic CA1 pyramidal cell was found to prevent the initiation of LTP. High-frequency stimulation of CA1 cells in the presence of this inhibitor resulted in only slight STP instead of LTP. These data suggest that both postsynaptic kinase activation as well as PKC activity are required for the initiation of LTP. Together with data presented above indicating that phorbol ester-mediated potentiation is mechanistically different from LTP, it appears likely that PKC is involved in 2 different mechanisms of synaptic potentiation -- phorbol-ester mediated potentiation and LTP. Furthermore, these results implicate PKC in the generation of STP as well as LTP, as STP is partially inhibited by application of the PKC inhibitor PKCI (19-31).

The role of PKC in the expression of LTP has been highly controversial. Application of sphingosine, which blocks kinase activation, was reported to have no effect on LTP expression once LTP has been established in a pathway, suggesting that kinase activation is not required for the expression of established LTP (Malinow et al., 1988). But application of H-7, which blocks kinase activity, was reported by at least some studies to inhibit LTP expression (Malinow, 1988; Wang and Feng, 1992). Malinow et al. found that application of H-7 reverses

established LTP, whereas an independent, unpotentiated pathway in the same slice is unaffected by the drug. Furthermore, the effect of H-7 appears to be reversible -- after washout of H-7, synaptic strength returns to the potentiated level -- suggesting that PKC activity is not required for the maintenance mechanism underlying LTP, but only for LTP expression. However, two more recent studies contradict Malinow et al. (1988) and indicate that H-7 inhibits unpotentiated pathways and potentiated pathways equally well (Muller et al., 1990; Perkel and Nicoll, 1991). These studies implicate kinase activity in the regulation of basal synaptic transmission rather than in LTP expression. It is possible that these contradictory results are due to differences in the batches of H-7 employed.

Although controversial, the results of Malinow et al. (1988) implicate a constituitively active or pre-activated kinase in the expression of LTP, because de novo kinase activation does not appear to be necessary for LTP expression. It had been shown previously (Kishimoto et al., 1983) that proteolytic digestion of PKC in vitro could generate a constituitively active kinase product, and the possibility that similar proteolytic events occur in LTP expression appears plausible (Malinow et al., 1988; it is possible that other, non-specific drug effects could underlie the differential effects of H-7 and sphingosine on LTP expression). More recently, two studies have presented evidence in favor of a role for a constituitively active PKC in LTP (see Schwartz, 1993). Klann et al. (1991, 1993) present evidence that LTP correlates with a persistent increase in activator-independent or constitutive PKC activity. More evidence for the production of a constituitively active PKC comes from Sacktor et al. (1993), who detect an increase in a proteolytic fragment of the $\zeta$ isotype of PKC, termed PKM$\zeta$, during the induction phase of LTP. However, it remains to be seen

whether the increase in PKMζ observed results from increased proteolysis or increased expression of PKCζ. This is a significant point because LTP (at least of the form described here that lasts for hours) does not require new protein synthesis (Frey et al., 1988), and therefore an increase in the expression of PKCζ alone is not likely to be relevant to the maintenance of LTP.

An important issue regarding the possible role of a constituitively active protein kinase in LTP maintenance is whether this kinase functions in the presynaptic or postsynaptic neuron. To address this issue, Malinow et al. (1988) applied H-7 intracellularly to a postsynaptic cell in a pathway that had undergone LTP, and found that this has no effect on the expression of LTP, even at concentrations high enough to block the induction of LTP. As Malinow et al. claim that H-7 applied to the whole slice blocks the expression of LTP, this finding implicates a presynaptic, constituitively active protein kinase in the expression of LTP (Fig. 1C). In contrast, Wang and Feng (1992) found that mixtures of PKC antagonists could block the expression of LTP, even when applied intracellularly to the postsynaptic cells alone. These results do not necessarily contradict those of Malinow et al., as it is possible that both postsynaptic PKC and presynaptic PKC play a role in LTP expression. Different kinase inhibitors may block LTP expression presynaptically or postsynaptically, suggesting that different kinases or kinase isotypes mediate these effects.

The studies described thus far are consistent with a model (Fig. 1B) in which protein kinase C directly responds to the calcium signal during the course of LTP induction and relays this signal to unknown substrates. However, it should be noted that all of the studies cited above are equally consistent with a

role for PKC in modulating cellular properties that enable or alter LTP induction rather than in the induction of LTP per se (see Perkel and Nicoll, 1991).

**Modulation of LTP**. The regulation or modulation of LTP may play a role in learning and memory (Shors et al., 1990; Abeliovich et al., 1993b). Activation of a number of G protein-coupled receptors has been found to modulate LTP, at least under certain conditions, including metabotropic glutamate receptors, serotonin receptors, and opioid receptors. Furthermore, activation of some G protein-linked receptors, such as metabotropic glutamate receptors, may be required for LTP induction. PKC appears to mediate some of these signals. This does not necessarily preclude a role for PKC in the NMDA receptor-mediated signal transduction pathway -- perhaps PKC mediates the two signals in concert.

A number of G protein-coupled receptors have been implicated in LTP induction and its modulation. Some of these receptors can induce phosphoinositide turnover through G protein activation of phospholipase C (Tanabe et al., 1992), and the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C forms 1,2-diacylglycerol, an activator of PKC, and inositol 1,4,5-trisphosphate, which induces calcium mobilization from intracellular stores and could thereby also activate PKC (Nishizuka, 1992). Metabotropic glutamate receptors, a family of G protein-coupled receptors present in both presynaptic and postsynaptic membranes (Tanabe et al., 1992), appear to be involved in LTP induction and modulation, perhaps through the activation of PKC. At least three metabotropic receptor isoforms (mGluR1$\alpha$, mGluR1$\beta$ and mGluR5) appear to be coupled to phosphoinositide turnover through G protein activation of phospholipase C, and are therefore likely to signal PKC activation (Tanabe et al., 1992). An inhibitor of metabotropic receptors, (RS)-$\alpha$-methyl-4-

carboxyphenylglycine (MCPG), blocks the induction of LTP (Bashir et al., 1993), and an activator of certain metabotropic receptor isotypes, aminocyclopentane dicarboxylate (ACPD), potentiates LTP (Aniksztejn et al., 1992). These results implicate metabotropic glutamate receptors in the induction of LTP. Other G protein-linked receptors, such as serotonin or opioid receptors, may also modulate LTP induction (Richter-Levin and Segal, 1991; Shors et al., 1990).

It is unclear whether G protein-linked receptor action in LTP is presynaptic, postsynaptic, or both. A possible postsynaptic role for G protein-linked receptors would be to activate PKC in synergy with calcium influx through NMDA receptors. PKC would thus serve as a "molecular coincidence detector" (Bourne and Nicoll, 1993) at active synapses (Fig. 2A), as calcium and diacylglycerol synergistically activate PKC (Shinomura et al., 1991).

Alternatively, G protein-linked receptor activation of PKC may serve to modulate postsynaptic NMDA receptor signaling events. In support of this view, Aniksztejn et al. (1992) found that the metabotropic glutamate receptor agonist, ACPD, can induce the up-regulation of NMDA receptor currents selectively, and they postulate that this mechanism may alter the threshold of LTP (Ben-Ari et al., 1992). Furthermore, they found that the PKC inhibitory peptide PKCI(19-36) blocks this effect, demonstrating a role for PKC in this mechanism. It is unclear whether synaptic NMDA receptor currents can be up-regulated by this mechanism, however, as Aniksztejn et al. (1992) apply NMDA to the bath, rather than stimulate Schaffer collateral fibers to evoke responses. G protein-coupled opioid receptors also appear to be capable of modulating NMDA receptor function. At the trigeminal nucleus, opioid receptor stimulation appears to alter NMDA receptor voltage-gating properties and to thereby increase the likelihood

of receptor activation (Chen and Huang, 1991, 1992; Markram and Segal, 1992). In another study implicating postsynaptic G protein-mediated signaling in LTP, lithium, which blocks G protein-coupled receptor signaling, was found to block LTP induction when injected into the postsynaptic cell (Ballyk and Goh, 1993). Cerebellar heterosynaptic long-term depression (cLTD) is an unrelated system that points to a role for postsynaptic metabotropic glutamate receptors in synaptic modulation, as cLTD appears to require both postsynaptic metabotropic receptor stimulation and postsynaptic PKC (Linden et al., 1991b, 1991a). In summary, there is some indirect evidence that postsynaptic metabotropic receptors can modulate LTP, but there is little direct evidence that NMDA receptor modulation through G protein-linked receptors is relevant to LTP at Schaffer collateral-CA1 pyramidal cell synapses.

In addition, there is evidence for a presynaptic role for G protein-linked receptors in LTP. In fractionated synapse preparations (synaptosomes), PKC activation by phorbol esters can increase glutamate release in response to depolarization. This effect may result from PKC-mediated inhibition of delayed-rectifier potassium channels resulting in a longer action potential, and thereby greater calcium influx through non-inactivating voltage-gated calcium channels (Fig. 2B; Barrie et al., 1991). The mechanism of potassium channel inactivation may involve either direct phosphorylation of the channel by PKC, or an induction of other second messenger effectors such as tyrosine kinases (Huang et al., 1993). Herrero et al. (1992) reported a similar increase of glutamate release in response to the metabotropic glutamate receptor agonist trans-(+/-)-1-Amino-1,3-cyclopentanedicarboxylic acid (ACPD). The effect of ACPD can be blocked by the PKC antagonist staurosporine or by the extended application of phorbol esters to down-regulate PKC, suggesting that the effect is mediated through

PKC. Interestingly, the effect of ACPD also requires arachidonic acid, a potent activator of PKC which can act synergistically with diacylglycerol to further activate PKC (Shinomura et al., 1991), and which has been implicated as a possible retrograde intercellular messenger in LTP (see Bliss and Collingridge, 1993). Increased glutamate release from depolarized synaptosomes, induced by metabotropic glutamate receptor-mediated activation of PKC, would establish a positive feedback loop, since additional glutamate release should further activate metabotropic glutamate receptors. Therefore, this provides a hypothetical mechanism to underlie LTP maintenance. However, it remains to be shown that metabotropic receptor activation can potentiate glutamate release at synapses between intact neurons.

In fact, application of ACPD depresses synaptic transmission at the Schaffer collateral-CA1 pyramidal cell synapse, suggesting a more complex role for metabotropic receptors. Furthermore, a variety of G protein-linked receptors including metabotropic glutamate receptors, $GABA_B$ receptors, adenosine receptors, and muscarinic acetylcholine receptors inhibit synaptic transmission by inhibiting presynaptic calcium channels and thereby reducing calcium influx during depolarization (Swartz, 1993). For example, presynaptic metabotropic signaling at glutamatergic synapses depresses basal synaptic transmission, a form of autoreceptor-mediated negative feedback. Swartz et al. (1993) found that in CA3 pyramidal cells activation of metabotropic glutamate receptors with the agonist ACPD leads to inhibition of N-type calcium channels. This suggests that metabotropic autoreceptors can depress glutamate release from CA3 cells through the inhibition of N-type calcium channels. Interestingly, phorbol esters block the metabotropic autoreceptor-mediated depression of synaptic current as well as N-type calcium current inhibition. This suggests that PKC blocks the

effect of metabotropic receptor inhibition, either at the N-type calcium channel or at another stage in the G protein signaling pathway. In favor of the latter model is the finding that phorbol esters can inhibit metabotropic receptor-mediated activation of phospholipase C (Aramori and Nakanishi, 1992), and that PKC can phosphorylate and thereby inhibit the function of G proteins directly (Katada et al., 1985; Pyne et al., 1989). Clearly, the hypothetical roles described above for G protein-linked receptors in LTP -- activating synaptic transmission through the inhibition of potassium channels and inhibiting synaptic transmission through the inhibition of calcium channels -- have opposite effects, and the relative contributions of these two mechanisms remains unknown.

It is tempting to think that a block in the inhibition of presynaptic N-type calcium channels (as described above), resulting in increased glutamate release, plays a role in LTP (Fig. 2C). Furthermore, a model of how G protein-linked PKC activation might modulate LTP is found in the modulation of a different synapse in the hippocampus: the synapse between mossy fiber axons, which arise from dentate granule cells, and CA3 pyramidal cells. Opioids have been found to modulate a different, NMDA receptor-independent form of long-term potentiation (mLTP; Weisskopf et al., 1993) at mossy fiber-CA3 synapses. The endogenous opioid peptide dynorphin appears to inhibit mLTP through $\kappa$ opioid receptors, G protein-coupled receptors, since a specific antagonist of these receptors, nor-binaltorphimine (nor-BNI), disinhibits mLTP. Although the exact mechanism for this inhibition is not clear, dynorphins are known to modulate presynaptic N-type calcium channels in several systems (Gross and MacDonald, 1987; Bean, 1989).

However, there is one experimental result indicating that a similar process involving G protein-coupled receptor stimulation does not underlie the modulation of LTP at Schaffer collateral-CA1 pyramidal cell synapses. Swartz et al. (1993) found that phorbol esters alone were able to activate N-type calcium channels, even in the absence of the metabotropic agonist ACPD, suggesting that N-type calcium channel activation is the mechanism that underlies phorbol ester-mediated synaptic potentiation. And, as mentioned above, LTP and phorbol ester-mediated synaptic potentiation are likely to act via different mechanisms (Gustafsson et al., 1988; Muller et al., 1990). Thus, if modulation of N-type calcium channels underlies LTP, it is necessary to hypothesize that phorbol esters mediate multiple independent functions. In other words, phorbol ester-mediated potentiation would have to be mechanistically different from phorbol ester-mediated activation of N-type calcium channels. Alternatively, phorbol esters and LTP may both modulate N-type calcium channels, but in mechanistically different ways.

PKC has been implicated in additional forms of receptor desensitization, such as desensitization of substance P-mediated inhibition of M current, a voltage-gated potassium current in frog sympathetic ganglion cells (Bosma and Hille, 1989), and desensitization to immunoglobulin receptor stimulation in B lymphocytes (Cambier et al., 1988). Thus, PKC activation may modulate LTP through alternative receptor desensitization mechanisms.

**PKC substrates in LTP.** In contrast to the substantial knowledge concerning the activation of PKC in LTP, opinions on the nature of downstream substrates of PKC remain largely speculative. A short list includes receptors

such as NMDA and AMPA glutamate receptors, other signal transduction molecules, and molecules involved in synaptic vesicle release.

The most obvious candidate for a substrate during the induction of LTP is the NMDA receptor, as it is the central player in LTP induction. As mentioned above, some isotypes of the NMDA receptor can be modulated by PKC (Kutsuwada et al., 1992; Urushihara et al., 1992; Kelso et al., 1992; ; Chen and Huang, 1992; Markram and Segal, 1992). In the trigeminal nucleus, activation of PKC by opioids can increase the probability of NMDA receptor channel opening, presumably due to channel phosphorylation (Chen and Huang, 1991). Furthermore, direct application of PKC to membranes of trigeminal neurons can alter the voltage gating properties of NMDA receptors so that they are significantly less likely to be blocked by magnesium at any given potential (Chen and Huang, 1992). However, Markham and Segal (1992) observe the opposite effect in the hippocampus -- depression of NMDA receptor channel current in response to phorbol ester application. These different results may reflect differences in PKC or NMDA receptor isotype expression between the trigeminal nucleus and the hippocampus. Metabotropic receptor stimulation may also lead to NMDA receptor activation, at least under certain conditions, and this process appears to be mediated by PKC (Aniksztejn et al., 1992). Thus, PKC might serve as a positive or negative feedback loop, becoming activated by calcium influx through the NMDA receptor, and in turn activating or inactivating the NMDA receptor (Fig. 3A). Another candidate PKC target is the AMPA class of glutamate receptors, as these receptors are primarily responsible for the EPSC in the course of normal transmission at Schaffer collateral fiber-CA1 pyramidal cell synapses. However, there is no evidence that modulation of these receptors by PKC takes place in the context of LTP.

Other channel proteins are also potential targets of PKC in LTP. Potassium (Baraban et al., 1985; Malenka et al., 1986), calcium (Rane et al., 1989), sodium (Numann et al., 1991), and chloride ( Madison et al., 1986) channels have all been shown to be modulated by PKC. PKC has been implicated in the inactivation of a calcium-activated potassium hyperpolarizing current (Baraban et al., 1985; Malenka et al., 1986) as well as a voltage-gated chloride channel (Madison et al.,1986). Because these two channels normally reduce the excitability of neurons, inactivating them would be expected to increase neuronal excitability and perhaps modulate the induction of LTP. Tetraethylammonium (TEA), a potassium channel blocker, has been shown to effect a variant, NMDA receptor-independent form of potentiation termed LTPk (Aniksztejn and Ben-Ari, 1991). LTPk may involve calcium influx through voltage-dependent postsynaptic calcium channels that are activated by the TEA-induced depolarization. Thus, potassium channel modulation is a viable mechanism in LTP, although it is not clear whether LTPk is mechanistically similar to LTP. It is unlikely that potassium channels would be major postsynaptic targets of PKC in LTP, as the postsynaptic membrane potential is often clamped during LTP induction. However, alterations in presynaptic membrane properties remain viable hypothetical mechanisms for PKC action (Fig. 3B).

Other candidate substrates for PKC in LTP are molecules involved in other second messenger signaling pathways. Biochemical analyses in both cell homogenates and in hippocampal slices that have undergone LTP have identified GAP-43 (also known as neuromodulin, B-50, F1, pp46, and P-57) to be a major PKC substrate found primarily at presynaptic termini (Routtenberg et al.,

1985; Linden et al., 1988). The biochemical properties of GAP-43 suggest one possible model for its action. GAP-43 (Masure et al., 1986; Alexander et al., 1988) as well as two other major PKC substrates, MARCKS (Albert et al., 1984) and neurogranin (Baudier et al., 1987), all harbor a calmodulin-binding domain, and phosphorylation of these substrates by PKC lowers their affinity for calmodulin. It is possible that the released calmodulin then serves to stimulate calmodulin-dependent kinases such as calcium, calmodulin-dependent protein kinase II (CamKII) or calmodulin-dependent phosphatases such as calcineurin (Fig. 3C). Calmodulin has been found to be limiting in neurons, at least under certain conditions (Meyer et al., 1992). Furthermore, phorbol ester treatment of PC12 cells increases free calmodulin concentration, and this correlates with an increase in CamKII activity (MacNichol and Schulman, 1992). Signaling through both PKC and CamKII appears to be necessary for LTP (Malinow et al., 1989), and neither signal appears sufficient. It has not been established whether these two signals act in parallel or in series during the course of LTP. Interaction between PKC and CamKII adds an additional layer of complexity to signaling in LTP.

PKC has also been implicated in modulating tyrosine kinase-mediated signaling events. Stimulation of M1 muscarinic acetylcholine receptors depresses a time- and voltage- dependent potassium channel, the M-current, in the hippocampus and elsewhere (Brown and Adams, 1980). M1 muscarinic receptors are G protein-linked receptors that upon stimulation activate phospholipase C and thereby PKC (see Hille, 1992). There is evidence that modulation of potassium channels by M1 muscarinic stimulation is ultimately due to tyrosine phosphorylation of potassium channels (Huang et al., 1993) This suggests that PKC serves to activate tyrosine kinases, which in turn

phosphorylate potassium channels and inactivate them. Consistent with this hypothesis, phorbol esters can mimic M1 receptor stimulation and effect the same tyrosine kinase-mediated potassium channel inhibition. Thus, PKC, a serine-threonine kinase, may modulate tyrosine kinase-mediated signaling events. Tyrosine kinase activity appears to be necessary for the induction of LTP (O'Dell et al., 1991b). Furthermore, NMDA receptor activation leads to the stimulation of tyrosine kinase activity (Bading and Greenberg, 1991; Ginty et al., 1992). However, there is currently no direct evidence regarding PKC modulation of tyrosine kinase activity in LTP.

Another possible role for PKC in LTP is in the modulation of putative retrograde messenger systems. The leading candidate mechanisms for retrograde signaling currently are the production of nitric oxide by nitric oxide synthase (NOS), and production of carbon monoxide by heme oxygenase (Zhuo et al., 1993; Stevens and Wang, 1993). NOS activity appears to be required for the induction of LTP, as pharmacological inhibition of NOS prevents the initiation of LTP (O'Dell, 1991a; Schuman and Madison, 1991; Bohme et al., 1991). In mouse striatal neurons, there is evidence that NMDA receptor activation leads to production of nitric oxide by a mechanism that involves PKC (Marin et al., 1992). Activation of NOS through the NMDA receptor is sensitive to staurosporine, a PKC inhibitor, and furthermore, down-regulation of PKC after long-term treatment with phorbol esters blocks the activation of NOS. This suggests that a similar mechanism may underlie the role of PKC in LTP (Fig. 3D). PKC does indeed phosphorylate one isoform of NOS, but paradoxically, PKC activation with phorbol esters leads to inhibition of NOS activity in cells transfected with a NOS expression vector (Bredt et al., 1992). It is possible that the activity observed in striatal cultures is due to a NOS isotype different from the transfected clone. It is

interesting to note that the production of nitric oxide by striatal neurons through this pathway leads to activation of guanylate cyclase, suggesting a possible mechanism by which nitric oxide as a retrograde messenger might act on the presynaptic terminal. Furthermore, this implicates PKC in the regulation of cGMP-mediated signaling.

The final and most attractive potential site for PKC action in LTP is in the regulation of neurotransmitter release (see Kelley, 1993). Synaptic vesicles are believed to form two different pools at the presynaptic terminus: one pool is already docked at the presynaptic membrane and can readily fuse to it (in less than a millisecond) in a calcium-dependent manner, whereas a reserve pool of synaptic vesicles needs to be mobilized in order to reach the presynaptic membrane. Both vesicle fusion and vesicle mobilization may be modulated by PKC (Fig. 3E). Phorbol esters augment the probability of spontaneous neurotransmitter release in the hippocampus (Malinow and Tsien, 1991) and the magnitude of evoked neurotransmitter release at the neuromuscular junction (Shapira et al., 1987), suggesting that PKC can modulate neurotransmitter release. Antibodies against GAP-43, a major PKC substrate found in synaptic terminals, disrupt neurotransmitter release (Dekker et al., 1991). Additionally, GAP-43 and another major PKC substrate, MARCKS, bind actin and appear to have a role in mobilizing the actin cytoskeleton in processes such as secretion (Rosen et al, 1990; see Gordon-Weeks, 1989). As noted above, PKC may also modulate transmitter release indirectly, by altering channel properties and thereby calcium influx during an action potential.

**The Diversity of PKC Isotypes**

PKC appears to play a complex role in LTP. This complexity could be explained by unique functional roles of different PKC isotypes in the mechanisms underlying LTP and its modulation. PKC is a family of at least 10 isozymes encoded by at least 9 genes (see Nishizuka, 1988, 1992). The different PKC isotypes have been subdivided into classical, new, and atypical PKC species. The classical PKC isotypes -- $\alpha$, $\beta$I, $\beta$II, and $\gamma$-- are activated by calcium, diacylglycerol (or phorbol esters), and phospholipids. The new PKC isotypes -- $\delta$, $\varepsilon$, $\eta$, and $\theta$ -- are not sensitive to calcium, and vary in their activation by diacylglycerol and phospholipid. The atypical PKC isotypes -- $\zeta$ and $\lambda$ -- are not sensitive to calcium or diacylglycerol, and vary in their sensitivity to phospholipids. PKC diversity has similarly been observed in invertebrates, suggesting that PKC isozyme diversity is an important cellular phenomenon. Both Drosophila (Schaeffer et al., 1989) and Aplysia (Kruger et al., 1991) appear to possess multiple PKC isozymes, and there is evidence that different isozymes possess unique functions in these systems (Smith et al, 1991; Sossin et al., 1992).

Classical PKC genes share four highly conserved domains (Nishizuka, 1988). A cysteine rich domain at the amino terminus of each gene, termed the C1 domain, encodes a putative phospholipid and diacylglycerol binding domain (Ono et al., 1989; Kaibuchi et al., 1989). A second conserved domain, the C2 domain, appears to be responsible for the calcium sensitivity of these enzymes. A third domain, the C3 domain, probably harbors the ATP-binding site of the kinase, and a fourth domain, the carboxy-terminal C4 domain, is highly homologous to the catalytic domain of many protein kinases. New PKC isotypes lack the C2 domain, consistent with their insensitivity to calcium. Atypical PKCs lack the C2 domain as well as part of the C1 domain, and as predicted these

isozymes show only variable sensitivity to fatty acids. Studies on partially purified classical PKC isozymes demonstrate significant differences in the activation requirements of the different classical PKC isozymes (Shinomura et al., 1991; Blumberg et al., 1993). Furthermore, the different classical PKC isotypes appear to possess somewhat different substrate specificities, at least in cell-free systems (Sheu et al., 1990; Blumberg et al., 1993).

The vastly different expression patterns of the different classical PKC isotypes support the idea of functional diversity among the isotypes (see Nishizuka, 1988). PKCα appears to be universally expressed; the splice variants PKCβI and PKCβII are expressed late in development and in the adult with well-defined tissue distributions that include expression in the CNS; PKCγ is expressed only in neurons of the adult CNS, and has therefore been implicated in neural-specific functions. Classical PKC isotype expression within the CNS is quite diverse: PKCβI, PKCβII, and PKCγ appear to have distinct but overlapping expression patterns. Perhaps the most striking patterns of expression appear in the cerebellum (Ase et al., 1988): PKCγ expression is restricted to Purkinje neurons, whereas PKCβI is expressed primarily in granule cell neurons, and PKCβII expression is restricted to the molecular layer. The subcellular distribution of classical PKC isotypes in the cerebellum also appears somewhat diverse. Immunoelectron microscopic analysis indicates that PKCγ is present at both presynaptic terminals and postsynaptic dendritic structures (Kose et al., 1988). Histochemical analysis indicates that PKCβI is present throughout the granule cell body, whereas PKCβII is concentrated in presynaptic axon termini that contact Purkinje cell dendrites (Ase et al., 1988). This data suggests that PKCγ might serve both presynaptic and postsynaptic functions in the cerebellum, whereas PKCβII may function strictly presynaptically. In the hippocampus, the

classical PKC isotypes α, βII, and γ are expressed at approximately equal levels, whereas βI is at expressed at a low level (Ase et al., 1988). Unfortunately, extensive immunohistochemical analysis of the subcellular localization of the different PKC isotypes in the hippocampus has not been performed. Biochemical fractionation data, in which isozyme distributions were measured in partially purified presynaptic terminals, indicates that PKCγ may not be present at presynaptic terminals in the hippocampus, suggesting that PKCγ may function strictly postsynaptically (Shearman et al., 1991). However, this data may not reflect the localization of PKCγ in vivo. In summary, there is indirect evidence that PKCβII may function strictly presynaptically in LTP, and that PKCγ may function either strictly postsynaptically or both presynaptically and postsynaptically.

**The Analysis of PKCγ Mutant Mice**

Mice mutant in the γ isotype of PKC have shed some light on the functional specialization of PKC isotypes. Abeliovich et al. (1993a) find that PKCγ mutant mice display altered LTP, whereas basal synaptic transmission and other forms of synaptic modulation are unimpaired. Conventional LTP is deficient in PKCγ mutant mice, but when high-frequency stimulation for LTP is preceded by a priming low-frequency stimulation, apparently normal LTP is observed. This suggests that PKCγ functions to modulate LTP or the likelihood of LTP, rather than function directly in the signaling cascade of LTP induction. PKCγ may not be required for at least some forms of PKC signaling in LTP, implicating other PKC isotypes in LTP. However, there is no evidence that the effects of PKC inhibitors on LTP differs from the effect of the PKCγ mutation on LTP, and therefore it remains to be seen whether other PKC isoforms function in LTP. To

this end, an unresolved question is whether PKC inhibitors can block the "primed LTP" which is unimpaired in PKCγ mutant mice. If "primed LTP" is impaired by PKC inhibitors, then PKC isotypes other than γ are most likely involved in LTP.

It is possible that certain PKCγ functions are redundant and can be performed by other PKC isotypes. However, no compensatory overexpression of PKCβ isotypes was observed in whole brain preparations from PKCγ mutant mice, as measured by total PKC activity or by western blotting with antibody to PKCβ. It is nonetheless possible that other isozymes are present at sufficiently high levels to partially complement the PKCγ mutation. This issue will undoubtedly be addressed by the production of mice mutant at other PKC genes.

The impaired regulation of LTP in PKCγ mutant mice suggests that PKCγ might normally function in one of the mechanisms hypothesized to modulate LTP as described above. For example, one hypothesis is that PKCγ conveys a signal from G protein-linked receptors such as metabotropic glutamate receptors to unknown substrates (Fig. 4). This can be investigated in PKCγ mutant mice by testing both postsynaptic G protein-linked receptor functions, such as the modulation of NMDA receptors, as well as presynaptic G protein-linked receptor effects, such as the activation of N-type calcium channels. NMDA receptor function appears to be intact in PKCγ mutant mice, but it remains a possibility that NMDA receptor modulation is aberrant in PKCγ mutant mice. It would be informative to test whether other reported modulatory effects on the induction of LTP (Coan et al., 1989; Fuji et al., 1991; Larson and Lynch, 1986) are affected by the PKCγ mutation.

PKC is known to modulate the activity of other second messenger systems, and thus an alternative hypothesis is that PKCγ serves to modulate other second messenger signals, either postsynaptically or presynaptically (Fig. 4). It would be of interest to test whether other intracellular second messengers systems, such as CamKII or tyrosine kinases, function normally in PKCγ mutant mice. To address this point initially, the production of different intracellular second messengers, different intercellular second messengers, and kinase activation in response to LTP induction or NMDA receptor stimulation could be measured in PKCγ mutant mice (Bading and Greenberg, 1991; Ginty et al., 1992). It would also be informative to analyze mutant mice harboring multiple mutations in PKCγ as well as either CamKII (Silva et al., 1992a, 1992b) or the tyrosine kinase fyn (Grant et al, 1992). If these kinases function through separate mechanisms, then double mutants might show a more severe phenotype than single mutants. Finally, PKCγ may modulate synaptic vesicle release and thereby alter LTP. Consistent with this hypothesis, PKCγ mutant mice appear to possess a slight enhancement in paired-pulse facilitation, another presynaptic phenomenon that is mechanistically different from LTP (see below). However, there is no direct evidence for the modulation of synaptic transmitter release by PKC in the course of LTP.

A limitation to the use of mutant mice for studies of LTP signal transduction has been the inability to induce or reverse a mutant phenotype acutely. Therefore, it has not been possible to exclude various sorts of indirect developmental deficits as the cause of LTP alteration in PKCγ mutant mice rather than a physiological alteration. However, there is no evidence for a developmental deficit in PKCγ mutant mice, and furthermore the specificity of the LTP deficit observed makes a developmental deficit less likely. One way to

exclude a developmental deficit in PKCγ mutant mice would be to complement the mutation acutely in hippocampal slices. To this end, it may be possible to generate a recombinant adenovirus that harbors a functional PKCγ gene, and to infect mutant hippocampus slices with this virus in hope of complementing the mutation and rescuing LTP (Davidson et al., 1993; Akli et al., 1993). This approach may also allow for the selective rescue of PKCγ expression in presynaptic or postsynaptic terminals of the Schaffer collateral-CA1 pyramidal cell synapse, and therefore may reveal the site(s) of PKCγ action.

A second limitation in studies of LTP with PKCγ mutant mice is that this approach cannot directly address whether PKCγ functions during the induction of LTP or in an enabling mechanism prior to LTP induction. The same limitation applies to pharmacological approaches that have been taken to study the role of PKC in LTP (see above). The interpretation that PKCγ functions in an enabling mechanism prior to LTP induction is suggested by the fact that apparently normal LTP can be observed if mutant slices are primed with a low-frequency stimulation prior to LTP induction.

PKCγ mutant mice appear to display a slight but consistent enhancement in paired pulse facilitation (PPF) compared to wild type mice. PPF is believed to be a presynaptic phenomenon, and this implicates PKCγ in a presynaptic role (although it is possible to conceive of models in which a postsynaptic kinase would effect a presynaptic event through retrograde signaling). A similar but more robust PPF phenotype was observed with mice deficient in the presynaptic protein synapsin I (Rosahl et al., 1993). PPF does not occlude or interact with LTP, suggesting that these two forms of synaptic modulation function via different mechanisms. Thus, the role of PKCγ in the modulation of LTP may well be

unrelated to a possible role in PPF. Preliminary data (A.A. and Y. G.) indicates that another form of synaptic modulation, phorbol ester-mediated potentiation, is intact in PKCγ mutant mice. Thus, PKCγ does not appear to be required for certain forms of PKC-mediated synaptic modulation.

**PKC and synaptic plasticity in invertebrates**

Signal transduction in the modulation of synaptic transmission has been investigated at defined synapses of two invertabrate species: Aplysia and Hermissenda. Because of the relatively simple neuroanatomy of these organisms, it is possible to study presynaptic and postsynaptic phenomena in defined neurons, and furthermore, it is feasible to correlate physiological changes with simple forms of learning. Strikingly, cellular and molecular mechanisms implicated in simple learning in invertebrates appear quite reminiscent of the mechanisms that have been implicated in the mammalian hippocampus.

In Aplysia, pleural ganglia sensory neurons that innervate the tail display plasticity associated with sensitization of the gill syphon-withdrawal reflex (see Kandel and Schwartz, 1982). This plasticity can be induced by the neurotransmitter serotonin, and results in several marked changes in sensory neuron properties, including decreased resting potential, increased duration of action potentials (spike broadening), enhanced excitability, and enhanced transmitter release. Serotonin responses in Aplysia sensory neurons appear to be mediated by both cAMP-dependent protein kinase (PKA) and PKC (Sugita et al, 1992). These two kinases appear to cooperate in this signal transduction mechanism in a complex and somewhat redundant fashion. PKA appears to be responsible for the enhanced excitability and depolarization of the sensory

neurons, and may also be responsible for an early component of the slowly-developing spike broadening observed in response to serotonin. PKC appears to be primarily responsible for a late stage of the slowly-developing spike broadening in response to serotonin, and this effect may be due to modulation of a voltage-dependent potassium current. Interestingly, both kinases appear to be involved in mediating serotonin-induced facilitation of synaptic transmission, but the relative contribution of each kinase appears to be dependent on the initial state of the synapse. This is reminiscent of the role of PKCγ in LTP, as it appears that the contribution of PKCγ to LTP is dependent on the initial state ("primed" or not "primed") of the Schaffer collateral-CA1 synapses (Abeliovich et al., 1993).

To address the roles of different Aplysia PKC isozymes in sensitization and in physiological response of sensory neurons, Sossin and Schwartz (1992) developed an assay for activity of classical PKC isotypes, which are calcium-dependent, and for activity of new or atypical PKC isotypes, which are calcium-independent. They found that serotonin application to sensory neurons results in the activation of classical, calcium-senstive PKC activity specifically, whereas calcium-independent PKC activity is not affected. These results indicate a functional segregation among the different PKC isotypes in Aplysia. Furthermore, they suggest that certain PKC isotypes may be dedicated to certain physiological functions. Finally, it is striking that stimulation of G protein-coupled receptors such as serotonin receptors in Aplysia and metabotropic receptors in the mammalian hippocampus may utilize a similar signaling pathway, namely classical PKC isozymes, to effect a change in presynaptic channel function that may underlie behavioral change.

Signal transduction has been investigated in another cellular correlate for behavioral change: the suppression of phototaxis in the marine mollusc Hermissenda (Crow, 1988). This behavioral effect also appears to be mediated by serotonin. Serotonin-induced enhancement of light-elicited action potentials in identified B photoreceptors of Hermissenda is essentially a presynaptic effect, as it is observed directly in light-activated cells. This effect appears to be mediated through PKC, as either phorbol esters or injection of purified PKC mimics this effect, whereas protein kinase inhibitors prevent it (Crow and Forrester, 1993). PKC activation leads to modulation of a calcium-dependent after-hyperpolarization potassium current and perhaps other potassium currents, and a correlated increase in the phosphorylation of potassium channels. Thus, PKC appears to mediate behavioral change in Hermissenda in a manner similar to its possible roles in Aplysia and in the mammalian hippocampus.

It is possible that the role PKCγ plays in LTP is very much in keeping with the role PKCs play in the serotonin receptor-mediated synaptic enhancements observed in Aplysia and Hermissenda. PKCγ appears to mediate a modulatory signal in LTP, perhaps in response to the stimulation of a G protein-coupled receptor such as the metabotropic glutamate receptor. As in the role of PKC in the invertebrate systems, PKCγ could mediate the modulation of potassium or calcium channels presynaptically to enhance action potentials and neurotransmitter release.

**Summary**

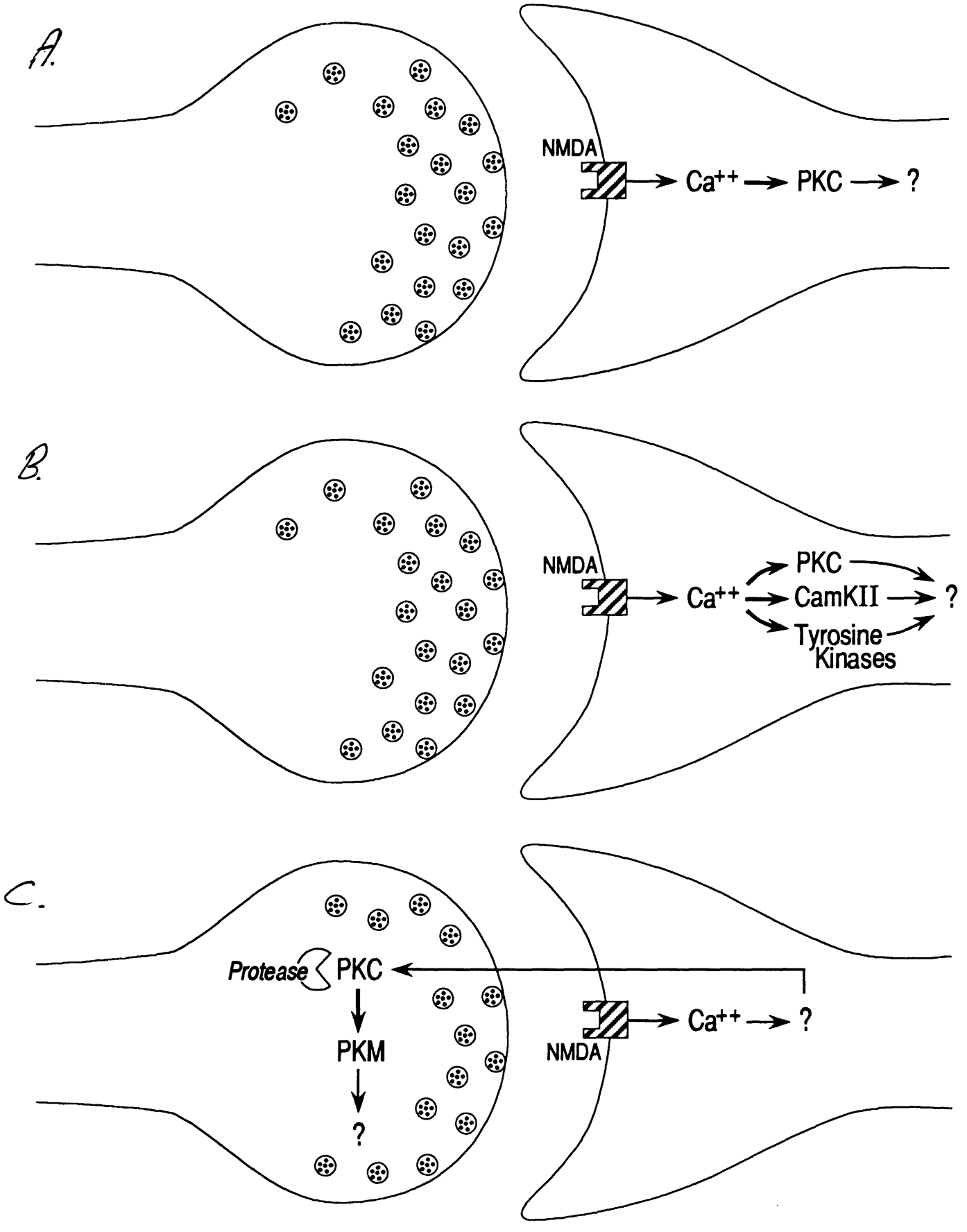
It is possible to derive a speculative model regarding PKCγ function in LTP (Fig. 4). It appears likely that PKCγ plays a role in the modulation or regulation of

LTP induction via G protein-mediated signaling events, perhaps involving the metabotropic glutamate receptor. PKCγ does not appear to be required for the transduction of the NMDA receptor-mediated calcium signal in LTP, as apparently normal LTP can be induced in hippocampal slices from PKCγ mutant mice if a low-frequency priming stimulus precedes high frequency stimulation. Furthermore, there is no evidence of a role for PKCγ in the maintenance of LTP. It is not clear whether PKCγ functions at the presynaptic terminal, the postsynaptic terminal, or both, in the course of LTP. Of central interest now is to determine the relevant substrates of PKCγ in LTP induction, and PKCγ mutant mice may prove useful in this regard.

Other PKC-mediated functions, such as phorbol ester-mediated potentiation, are most likely served by PKC isotypes other than PKCγ. PKCα is expressed constituitively and appears early in development, suggesting that this isotype may be dedicated to basic cell functions such as cell growth and differentiation. PKCβII is localized presynaptically, at least at certain synapses in the cerebellum, and it has been speculated that PKCβII may play a role in presynaptic functions such as phorbol-ester mediated potentiation. A proteolytic fragment of PKCζ has been implicated in the maintenance of LTP, but this remains highly speculative. The degree to which PKC isotypes function redundantly is not clear, but additional genetically altered mice should shed light on this issue. In summary, it is apparent that PKC not only underlies certain mammalian plasticity mechanisms, but is also a source of confusion.

# FIGURES

Figure 1. Hypothetical roles for PKC in the mechanism of LTP. A; a simple model is that PKC transduces NMDA receptor-mediated calcium influx to unknown target molecules in the course of LTP induction. B; PKC may be one of several second messengers that transduce the NMDA receptor-mediated calcium influx in the course of LTP induction. C; a controversial model that implicates a constituitively active PKC degradation product in the expression of LTP. There is some evidence in favor of a presynaptic role for such a molecule, and this invokes a retrograde messenger.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B.
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PKC
CamKII
Tyrosine Kinases
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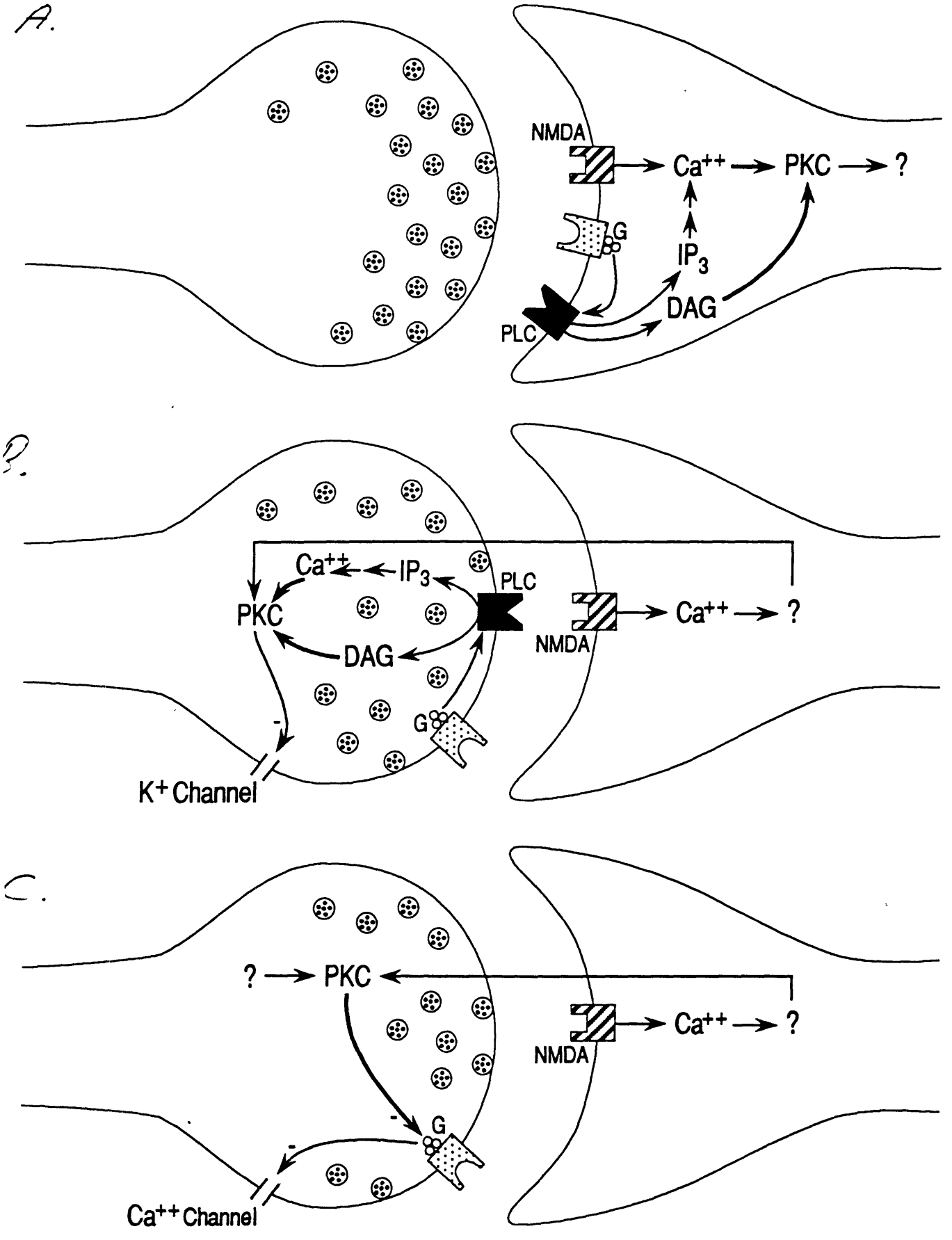
C.
Protease
PKC
PKM
?
NMDA
Ca++
?

Figure 2. Hypothetical roles for PKC in the modulation of LTP. A; PKC may transduce signals from postsynaptic G protein-linked receptors (G) such as the metabotropic glutamate receptor to unknown substrates. Additionally, PKC may serve as a coincidence detector for G protein-linked and NMDA glutamate receptor activation. B; PKC may transduce signals from presynaptic G protein-linked receptors (G) such as metabotropic glutamate receptors to calcium or potassium channels. C; PKC may block presynaptic G protein-linked receptor (G) -mediated inhibition of calcium channel function.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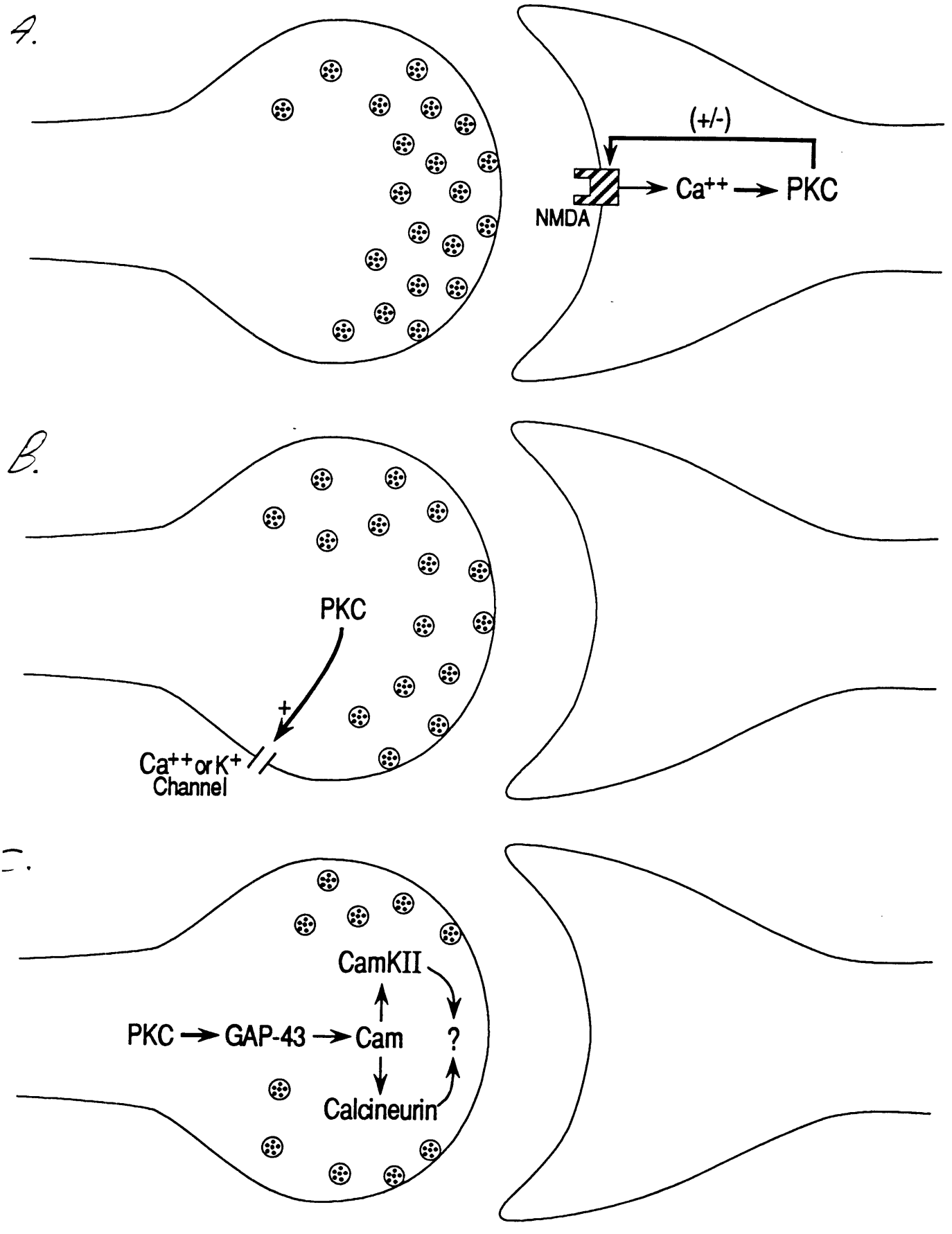
G
K+ Channel
C.
?
PKC
NMDA
Ca++
?
G
Ca++ Channel

Figure 3. Hypothetical PKC substrates in LTP. A; PKC modulation of NMDA receptor function may play a role in LTP. B; PKC may activate presynaptic calcium or potassium channels in the course of LTP induction. C; PKC may modulate other second messenger signaling pathways that are involved in the induction of LTP. Phosphorylation of GAP-43, a major presynaptic PKC substrate, lowers the affinity of GAP-43 for calmodulin, and the released calmodulin may activate other signaling molecules such as CamKII or calcineurin. D; PKC may modulate the production of putative retrograde messengers (RM) such as nitric oxide or carbon monoxide by phosphorylating enzymes such as nitric oxide synthase or heme oxygenase. E; PKC may modulate neurotransmitter release, either at the vesicle fusion step or at the vesicle mobilization step.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PKC
NMDA
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C.
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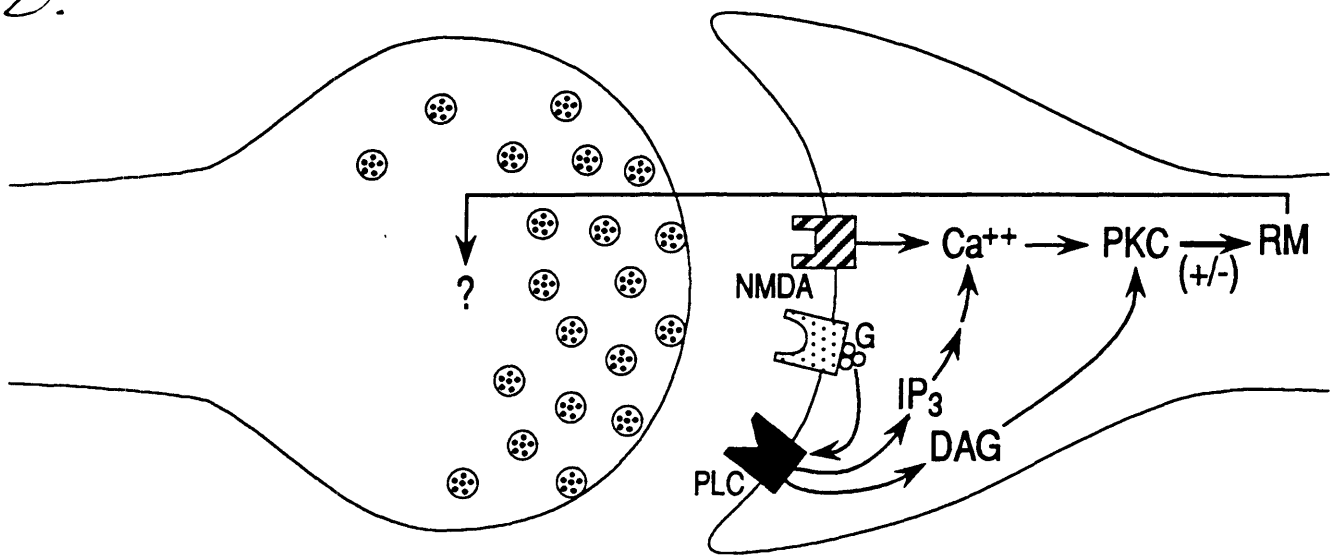
PKC
GAP-43
Cam
?
Calcineurin

D.




E.

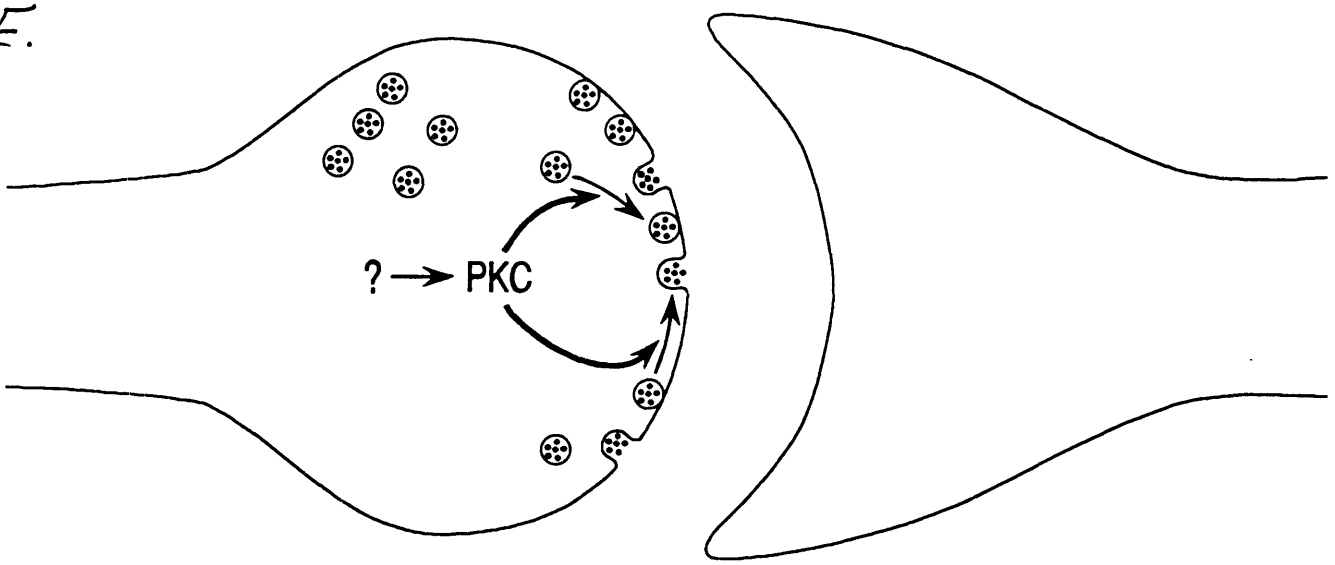
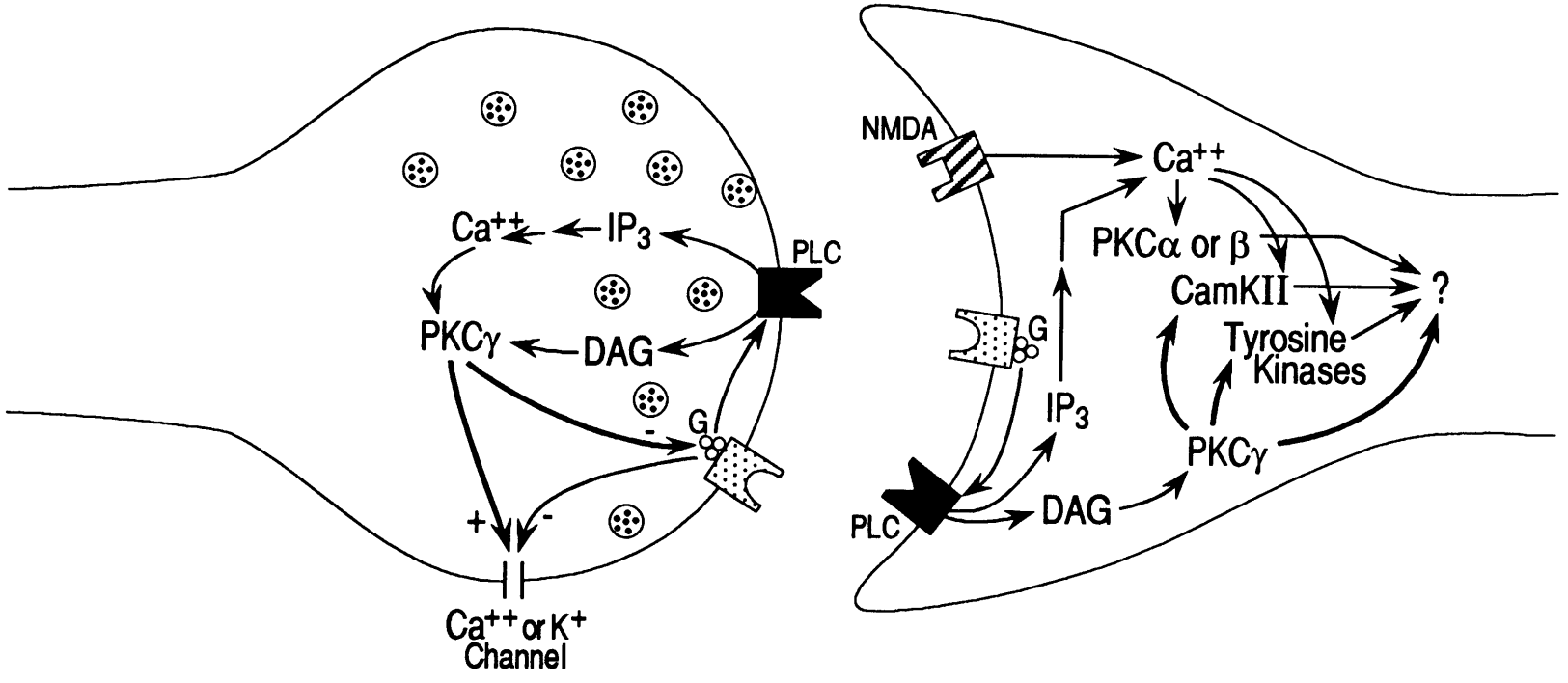


Figure 4. A speculative model regarding the role of PKCγ in LTP. PKCγ appears to be required for the modulation of LTP but may not be required for the basic mechanism of LTP signaling. PKCγ may uniquely mediate certain presynaptic or postsynaptic G protein-linked receptor (G) functions. Possible postsynaptic roles include the modulation of second messenger pathways and retrograde messenger synthesis. Possible presynaptic roles include the modulation of ion channel function, synaptic vesicle release, and G protein-linked receptor-mediated ion channel inhibition.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Channel
NMDA
Ca++
PKCα or β
CamKII
?
Tyrosine
Kinases
G
IP3
PKCγ
PLC
DAG

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# CHAPTER 2

# Modified Hippocampal Long Term Potentiation in PKCγ Mutant Mice

**Asa Abeliovich, Chong Chen, Yukiko Goda, Alcino J. Silva, Charles F. Stevens, and Susumu Tonegawa**

*Center for Cancer Research and Department of Biology
Massachusetts Institute of Technology
Cambridge, MA 02139

#Howard Hughes Medical Institute at the Center for Cancer Research and Department of Biology
Massachusetts Institute of Technology
Cambridge, MA 02139

Howard Hughes Medical Institute
Salk Institute
10010 North Torrey Pines Road
La Jolla, CA 92037

## SUMMARY

**Calcium-phospholipid-dependent protein kinase (PKC) has long been suggested to play an important role in modulating synaptic efficacy. We have created a strain of mice that lacks the γ subtype of PKC to evaluate the significance of this brain-specific PKC isozyme in synaptic plasticity. Mutant mice are viable, develop normally, and have synaptic transmission that is indistinguishable from wild type mice. Long-term potentiation is, however, greatly diminished in mutant animals while two other forms of synaptic plasticity, long-term depression and paired-pulse facilitation are normal. Surprisingly when tetanus to evoke LTP was preceded by a low frequency stimulation, mutant animals displayed apparently normal LTP. We propose that PKCγ is not part of the molecular machinery that produces LTP but is a key regulatory component.**

## INTRODUCTION

Kinases have been repeatedly implicated in the mechanism of long term potentiation (LTP), the most intensively studied cellular model for memory (see Bliss and Collingridge, 1993). Recently phosphatases have been found to participate in the inverse mechanism for the regulation of synaptic strength, hippocampal long term depression (LTD) of the sort defined by Dudek and Bear (Mulkey et al., 1993; Dudek and Bear, 1992). Although the addition and removal of phosphate groups on unidentified proteins is widely believed to be important in

the regulation of synaptic strength, very little evidence relates to the precise mechanisms. One possibility is that phosphorylation of some special protein(s) is an integral step in the pathway for LTP, but an alternative is that phosphorylation of this protein(s) is simply regulatory, so that the level of phosphorylation controls the likelihood, threshold, or magnitude of LTP.

Mutant mice produced by embryonic stem (ES) cell gene targeting technique to be defective in a particular gene product provide an attractive model system in which to study LTP. We can obviate the lack of highly specific pharmacological tools to study various protein kinases with this approach; furthermore, we can investigate consequences of the mutation for behavior (see for example Silva et al., 1992a; Silva et al., 1992b; Grant et al., 1992). Hippocampal LTP in particular has been suggested to play an important role in certain types of learning and memory and has attracted considerable attention (Bliss and Collingridge, 1993). The availability of knockout mice thus provides a unique opportunity to address the specific role of kinases in LTP, and the relationship between LTP and learning and memory processes.

In an earlier study, Silva et al. (1992a) demonstrated that a null mutation in one of the kinases frequently implicated in LTP, α-CaM Kinase II (αCaMKII), greatly diminishes the magnitude of LTP, but seems not to eliminate it entirely. The fact that some LTP persists in the αCaMKII knockout mice was interpreted as indicating that this enzyme plays a regulatory, not an essential role. But, as Silva et al. have pointed out, the alternative possibility is that some other kinase could substitute, although not as effectively.

We have continued the program of investigating the molecular substrates of synaptic plasticity by producing mice that lack the γ isoform of $Ca^{2+}$-phospholipid-dependent protein kinase (PKC). PKC constitutes a family of isoenzymes involved in signal transduction pathways in diverse systems. This enzyme was chosen for study because pharmacological studies have repeatedly implicated PKC as playing a role in LTP (for reviews see Schwartz, 1993; Ben-Ari et al., 1992). For example injection of PKC into hippocampal pyramidal cells elicits what may be aspects of LTP (Hu et al., 1987), and relatively selective inhibition of postsynaptic PKC blocks LTP induction (Malenka et al., 1989; Malinow et al., 1989; Reymann et al., 1988; Wang and Feng, 1992). PKC may also play a role in maintenance of LTP (Klann et al., 1991; Sacktor et al., 1993). In addition redistribution of subcellular PKC in hippocampal neurons appear to coincide with LTP (Akers et al., 1986). The γ isoform was selected because it is brain specific, is richly represented in hippocampus where LTP and LTD are robustly expressed (Nishizuka, 1988), and appears to be present primarily in the dendrites and cell body of neurons (Huang et al., 1988). Moreover the γ isoform appears late in development, so that brain defects that simply reflect abnormal neural development are less likely.

PKCγ mutant mice are viable, their behavior in usual situations is not noticeably impaired, the brain anatomy is not apparently disturbed, and synaptic transmission appears normal. LTD is intact, but LTP is aberrant. LTP, as induced by commonly used high frequency stimulation is greatly diminished. When this high frequency stimulation is given after a low frequency stimulation of the sort used to produce LTD, LTP of approximately normal magnitude can be elicited. The production of LTP does not depend on LTD actually appearing, but just on the prior stimulation history of the synapses; we thus hypothesize that

PKCγ is involved in regulating LTP, but is not required for the actual process of synaptic plasticity.

## RESULTS

### Generation of PKCγ mutant mice

PKCγ mutant mice were generated by homologous recombination into the germline utilizing embryonic stem (ES) cell technology. A homologous recombination vector was constructed containing PKCγ sequences harboring a 2.5 kb deletion. Integration of this vector would result in the loss of an exon containing the nucleotide binding domain required for catalytic activity (Figure 1A). This vector was transfected into E14 ES cells (Thompson et al., 1989), and clones containing the desired homologous integration were identified by G418 selection and Southern hybridization. Five positive clones were identified, and these were injected into blastocysts to produce chimeric animals. Chimeric males were mated to C57BL/6 females, and three chimeric animals transmitted the mutation through the germline. Heterozygote progeny were intercrossed, and the offspring were typed by Southern analysis for the PKCγ mutation (Figure 1B). PKCγ mutant progeny are viable and can only be distinguished from wild-type littermates in the normal cage environment by mild uncoordination (Abeliovich et al., 1993).

Northern blot analysis of RNA from wild type and PKCγ mutant mice (Figure 1C) demonstrated that PKCγ mutant mice lack normal PKCγ RNA transcripts. Western blot analysis of total brain protein with a rabbit polyclonal antiserum specific to PKCγ indicated that no PKCγ protein could be detected in brains of mutant mice (Figure 1D). Additionally, we did not detect up-regulation

of PKCβ, the major PKC isotype in the brain. Analysis of PKC activity in homogenates of mutant and wild type brains indicated that mutant mice possess a 29 ± 4% decrease in total PKC activity (data not shown), consistent with the known distribution of PKCγ in the brain (Sae et al., 1988). Furthermore, immunoprecipitation of PKCγ activity with a mouse monoclonal antibody indicated that mutant mice were devoid of immunoprecipitable PKCγ activity (data not shown).

Histochemical analyses of PKCγ mutant mice did not reveal any gross anatomical abnormalities in the hippocampus or elsewhere (n=3; Figure 2). This is consistent with the late onset of PKCγ expression (Nishizuka, 1988).

**Synaptic transmission is unimpaired**

We have examined the amplitude and time course of synaptic responses, the functioning of NMDA receptors, and paired pulse facilitation. The only difference we could detect between mutant and wild type slices is a very slight, but significant, increase in paired pulse facilitation.

Synaptic activity in hippocampal CA1 pyramidal cells is evoked by stimulating Schaffer collateral and commissural axons, and is recorded in two ways: (1) field potential recordings which monitor additive responses from a group of cells, and (2) whole-cell recordings that monitor responses from a single cell. In both field potential and whole-cell recordings, the evoked response in PKCγ mutant slices is indistinguishable from the wild type mice. In whole-cell recordings, the average synaptic current decay time constant for the mutant animals was 11.5 ± 0.5 msec, and for normal animals 11.1 ± 0.3 msec. Because response size depends on stimulus intensity, a comparison of the magnitude of

postsynaptic responses is more difficult. We have used the fact that, at sufficiently low stimulus intensities, only a single quantum of transmitter appears to be released most times (Raastad et al., 1992) -- so called "minimal stimulation" -- and have compared the response amplitudes and time courses to such minimal stimulation. Figure 3 compares the amplitude histograms of responses under minimal stimulation conditions between wild type and mutant cells. We could detect no systematic effect of PKCγ absence.

Synapses of hippocampal cells that exhibit LTP use the excitatory neurotransmitter, glutamate. LTP is caused by repetitive activation of presynaptic cell, and its onset is determined by an increase in the size of glutamate-dependent postsynaptic response, that is, postsynaptic membrane potential. Two types of glutamate receptors can be identified in the postsynaptic membrane; these receptors are classified by their differential sensitivity to glutamate analog, N-methyl-D-aspartate (NMDA). Studies with selective inhibitors indicate that prerequisite for LTP induction appears to be binding of glutamate to NMDA receptors but not to non-NMDA receptors. Glutamate binding alone, however, is not sufficient to activate NMDA receptors. NMDA receptor function is also dependent on the postsynaptic membrane potential. NMDA receptor activation and LTP induction, therefore, requires temporal coupling of glutamate binding and postsynaptic membrane depolarization (for review see Bliss and Collingridge, 1993).

We have, therefore, examined the NMDA component of synaptic currents by holding the membrane potential at -50 mV where sufficient number of NMDA receptors are active to produce substantial synaptic currents. An antagonist of non-NMDA receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), was

perfused to block the contribution of non-NMDA component. The time course, and magnitude of the NMDA receptor components, and the ratios of non-NMDA to NMDA receptor component of synaptic currents do not differ between wild type and mutant slices (data not shown). Further, the voltage sensitivity of the NMDA receptor that is critical for its activity, and consequently for LTP, is unchanged by the absence of PKCγ (Figure 4).

Paired pulse facilitation is a potentiation of the postsynaptic response to the second stimulus of the pair. It is caused by enhanced transmitter release from the presynaptic terminals (Mallart and Martin, 1968). Figure 5 presents data from experiments in which pairs of pulses were applied at varying interpulse intervals between 20 and 160 msec. The mutant slices showed paired pulse facilitation that is slightly, but consistently, above that of the wild type slices.

**LTD is unimpaired**

LTD is an important form of synaptic plasticity that shares steps with LTP in that both require the initial influx of Ca2+ ions through NMDA receptor channels. We have examined this form of synaptic plasticity and find it to be unimpaired in induction, maintenance and magnitude.

LTD was produced by the Dudek-Bear protocol (Dudek and Bear, 1992). Figure 6 presents histograms of the amplitude of LTD produced in 26 wild type and 29 mutant slices with field potential recording. As with LTP (see below), the magnitude of the effect is quite variable from slice to slice. The mean size, shape of the distribution function and variance of the distribution function are not significantly different for the wild type and mutant animals. Thus, LTD is present in the mutant animals that lack PKCγ.

**LTP is Abnormal**

The usual test for LTP is to examine the increase in synaptic strength produced by tetanic stimulation. We have carried out field potential experiments in 14 mutant slices to compare with 17 control slices. LTP was clearly deficient in mutant slices (Figures 7 and 8A). Identical results were obtained by an independent set of field potential recordings comparing LTP in 10 mutant and 4 wild type slices. Whole-cell recording is a more effective method for producing LTP than field potential recording since it allows a direct control of the postsynaptic membrane potential which is crucial for LTP induction. To test whether LTP deficiency in mutant slices can be overcome by controlling the postsynaptic membrane potential, whole-cell recordings have been carried out (7 control and 12 mutant slices). LTP clearly was attenuated or absent in the mutants (averaged trace not shown). To compare this effect quantitatively, we have made amplitude histograms (Figure 8). As usual, the amount of LTP produced follows a probability distribution with a standard deviation of about 8 to 30% (when LTP is expressed as % of baseline). The mutant animals are clearly deficient in LTP, but the relatively large standard deviations make it difficult to decide if LTP is entirely absent (although the mean of the distribution is clearly different between the mutant and control slices in both field potential and whole-cell recordings; see for example Figure 7).

In an attempt to see if LTP is present in some cases, we determined the probability distributions of LTP amplitude for mutant and wild type slices in the presence of 2-amino-5-phosphonopentanoate (AP5), an antagonist of NMDA receptors that disables LTP induction (Collingridge et al., 1983). The idea behind this experiment is that, if some LTP is present in the mutant slices, the probability

distribution should be shifted to the left (to smaller values). Also, when LTP is inhibited by AP5 treatment, distribution of synaptic responses to tetanic stimulation should be indistinguishable between the mutant and wild type animals. Figure 9 shows the relevant cumulative distributions of responses after high frequency stimulation, monitored in the presence of 50mM AP5. As is apparent, no difference can be detected between untreated mutant slices (n=14), AP5 treated mutant slices (n=10) and AP5 treated wild type slices (n=9).

We conclude that LTP is either absent or greatly diminished under the conditions of these experiments. LTP is a saturable phenomenon in that repeated application of tetanus cannot further the potentiation of synaptic response after a plateau is reached (Bliss and Collingridge, 1993). Because one form of synaptic plasticity (LTD) appears normal in the mutant animals, while another appears to be absent, we sought to test the hypothesis that LTP fails to appear because it is saturated in the slices when we obtain them from the animals. Under this hypothesis PKC$\gamma$ would perhaps regulate the relative balance between LTP and LTD. Were this true, the mutants should reveal LTP if preceded by the production of LTD: if we unsaturated LTP, we should then be able to elicit it.

Figure 10A displays the summary of recordings in which LTD was elicited prior to the production of LTP. After the LTD protocol, as is clear from this set of experiments, normal appearing LTP can be elicited in the mutant slices. Cumulative histograms from 21 mutant and 9 control slices (Figure 10B) reveal that significant LTP can be consistently produced in the mutant slices. When LTP was elicited in the presence of 50mM AP5 after the LTD protocol no potentiation was observed in the mutant slices; the mean response after the

tetanus (81 ± 6%; n=9) was not significantly different from the mean LTD response (87 ± 3%; n=29).

Although the prior LTD stimulation protocol does enable the subsequent production of LTP, the mechanistic explanation appears not to be that LTP was saturated: the magnitude of LTP is unrelated to the magnitude of the preceding LTD, whereas the saturation hypothesis would require that greater LTD would permit greater LTP. Throughout this manuscript LTD and LTP responses have been calculated as percentage of the very initial baseline before application of tetanus or low frequency stimulation with the exception of Figure 11A. In Figure 11A LTP response subsequent to LTD is displayed as percent potentiation relative to the mean response after LTD and is compared to LTD response expressed as percent depression relative to the initial baseline in order to test the following prediction: if the magnitude of subsequent LTP correlates with the magnitude of prior LTD then data points should fall on a line with slope of +1. Our results demonstrate that there is no correlation between the two magnitudes. Thus the quantity of LTP produced appears independent of the extent of LTD.

To test further the role of prior LTD in enabling LTP production, we applied 50 mM AP5 during the LTD protocol, and then washed out the AP5 for 30 minutes before using the standard tetanic stimulus to produce LTP. In these experiments, the LTD was, as we would expect, effectively blocked by AP5 (Figure 11B, compare lanes a and c), but the amount of LTP produced was not significantly different from that of control slices subjected to the same procedures in the absence of AP5 (Figure 11B, compare lanes b and d).

We conclude, then, that the history of low frequency stimulation does somehow enable the slices from mutant animals to express LTP, but that the mechanism by which the effect works is not through the actual production of LTD.

## DISCUSSION

The absence of the γ isoform of PKC has little detectable effect on baseline synaptic transmission. We could find only a very slight increase in paired pulse facilitation. Synaptic plasticity is, however, modified but not eliminated because LTD is apparently normal, and LTP -- which is absent under the usual test circumstances -- can be elicited if preceded by a period of low frequency stimulation.

Clearly, the action of PKCγ is not necessary for the production of LTP. We propose that this kinase is required for some regulatory role in LTP, although we cannot exclude the possibility that PKCγ action is in fact essential for LTP, but that some other kinase is replacing it. This hypothesis may be tested by making double deletions. For example, it is now possible to produce mice that are doubly deficient in PKCγ and αCaMKII. In such mutant animals would LTP be completely abolished? If kinases are regulatory then we should observe occasional LTP, albeit less frequently.

The fact that LTP is enabled by low frequency stimulation, but that the appearance of LTD or calcium influx through NMDA receptor channels seem irrelevant for this effect, surprised us. This observation indicates a complexity in the control of LTP inducing mechanisms that, as far as we are aware, has not been revealed in earlier studies, although reports of similar effects have

appeared earlier (Huang et al., 1992; Coan et al., 1989; Fujii et al., 1991). Interesting, however, the earlier investigations reported what, on the surface at least, seem to be an effect in the opposite direction: low frequency stimulation or weak tetani that elicit short-lasting potentiation have been found in several studies to inhibit the subsequent triggering of LTP for a period of time. Perhaps this inhibition is mediated through PKCγ action, and removing this regulatory pathway unmasks another enabling mechanism. In any case, we propose that the use history of a synapse modifies the state of LTP regulatory systems in ways that have not previously been apparent. Presumably such mechanisms have functional significance, but our study reveals too little about the properties of the postulated regulation to permit speculation about what these functional consequences might be.

An interesting observation is that the usual protocol for LTP production does not, in every circumstance, accurately assess the potential for a slice to produce LTP. In our PKCγ mutant mice one would conclude from the usual methods that the slices lack the capacity to produce LTP; our study, however, demonstrates that the hippocampal slices from the mutant mice retain the capacity to express LTP of approximately normal magnitude when an alternative stimulation protocol is used. It remains to be seen whether such a stimulation protocol and LTP induced by it have any physiological relevance in an intact animal.

## MATERIALS AND METHODS

### Mapping and cloning of mouse PKCγ

PKCγ was cloned from a 129/Ola mouse genomic cosmid library (Stratagene) with a rat PKCγ cDNA probe. The exon structure was determined in a region of PKCγ that encodes the ATP-binding motif, a required component of the kinase catalytic domain (Freisewinkel et al., 1991). Exon A corresponds to amino acids 363-426 of the rat protein (Knopf et al., 1986). The homologous recombination vector p21 was assembled in a tetramolecular ligation reaction, using a 4.5 kb PstI fragment of cosmid 3C 5' of exon A (subcloned into Bluescript II [Stratagene] and excised with BamHI and XhoI), a 1.8 kb XhoI-EcoRI fragment containing neo driven by the PGKI promoter (a gift from Dr. M. Rudnick), a 5.5 kb fragment of cosmid 3C 3' of exon A (subcloned into Bluescript II and excised with EcoRI and XbaI), and XbaI-BamHI restriction-digested Bluescript II vector. This construct was designed to delete a 2 kb fragment of the PKCγ gene, including exon A.

### Homologous recombination in mouse ES cells and introduction of the deletion into the germline

E14 ES cells (Thompson et al., 1989) were transfected with 50 mg of plasmid P21 (linearized with NotI restriction enzyme) by electroporation. (Biorad gene pulser set at 800V and 3mF). G418 selection (150 mg/ml) was applied 24 hours after transfection, and G418-resistant colonies were isolated on days 5-7 of selection. Genomic DNA isolated from approximately 200 colonies was digested

with BamHI restriction enzyme, Southern blotted, and hybridized with a 1.2 kb BamHI-PstI fragment of cosmid 3C 5' of the flanking region in p21 (Figure 1). Six clones were identified that harbored the predicted homologous integration. These clones were confirmed by digestion with a BamHI-PvuII probe within the 3' flanking region of p21 (Figure 1), or an NcoI-EcoRI fragment of cosmid 3C 3' of the flanking sequences in plasmid p21 (data not shown). Chimeric mice were generated as described by Bradley (Bradley, 1987). Germline transmission of the deletion was determined by Southern blotting of tail DNA as described above. Later, mice were typed by PCR analysis with a set of neo primers (5'-CTTGGGTGGAGAGGCTATTC-3' and 5'-AGGTGAGATGACAGGAGATC-3', a 280 bp fragment) and a set of primers to exon A (5'-GGATGACGATGTAGACTGCA-3' and 5'-CTCTTACCACTGGTCACATC-3', a 200 bp fragment).

**Analysis of PKCγ RNA**

RNA was isolated from total brain by extraction with guanidine HCl followed by cesium chloride gradient centrifugation. RNA (200 mg) was electrophoresed in the presence of formaldehyde, northern blotted, and probed with a 200 bp PCR product within exon A of PKCγ (see above) or a human glyceraldehyde 3-phosphate dehydrogenase (hG3DH, Clonetech) control probe. hG3PDH is a housekeeping gene that cross-hybridizes with the mouse gene and serves to control for RNA quantity.

**Western Blot**

5 mg of partially purified brain extract (Yasuda et al., 1990) was denatured in SDS loading buffer and separated by SDS-PAGE. Gels were then transferred to Hybond-ECL nylon membranes (Amersham), and membranes were incubated

with anti-PKCγ or anti-PKCb antibody (rabbit anti-rat peptide antibodies at 0.2 mg/ml, Gibco/BRL). Membranes were subsequently incubated with a goat anti-rabbit horseradish peroxidase-labeled antibody (Southern Biotechnology Associates, Inc.) Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham).

**Histochemistry**

Mice were perfused with 2% paraformaldehyde in PIPES buffer under deep sodium pentobarbitol anesthesia (anesthesia performed under the guidelines of the Massachusetts Institute of Technology Division of Comparative Medicine), and their brains were postfixed for up to 8 hours at 4°C and then soaked overnight in phosphate buffer containing 30% sucrose and 2 mM MgCl2 at 4°C. Transverse sections were cut at 30 or 40 μM on a sliding microtome or with a cryostat and were stained with cresyl violet.

**Electrophysiology**

Standard procedures were used to prepare transverse hippocampal slices from wild type or mutant mice (male or female, mostly 1 to 3 months old; younger animals were typically used for whole-cell recordings). Slices (350mm) were transferred to an incubation chamber and were allowed to recover for at least 1hr before recording. During recording each slice was submerged under a continuously perfusing medium that was saturated with 95% O2, 5% CO2. The perfusate contained: 120mM NaCl, 3.5mM KCl, 1.25mM NaH2PO4, 26mM NaHCO3, 1.3mM MgCl2 and 2.5mM CaCl2. Whole-cell patch clamp experiments were performed in the presence of 50μM picrotoxin. Experiments were carried out at room temperature (23-25°C). Schaffer collateral/commissural fibers were stimulated by bipolar tungsten electrodes that delivered 100msec

pulses. Field potentials were recorded in field CA1 with glass electrodes filled with perfusing medium. Whole-cell patch-clamp recordings were carried out according to standard techniques. Excitatory postsynaptic currents (EPSCs) were recorded with glass electrodes (~4MW) filled with 130 mM cesium gluconate, 5mM CsCl, 5mM NaCl, 10mM HEPES-CsOH pH 7.2, 0.5mM EGTA, 1mM MgCl2, 2mM Mg-ATP and 0.2mM Li-GTP. Stimulus intensity was adjusted to evoke baseline responses of similar sizes for all recordings. For "minimal stimulation" recordings in whole-cell mode stimulus intensity was adjusted such that failure rate of responses elicited was ~40%. Tetanus to evoke LTP consisted of 5 trains of 100Hz stimulation, each lasting 200msec at an intertrain interval of 10sec. In whole-cell recordings, the membrane potential of postsynaptic cell was held at -70mV except during tetanus when it was -30mV. LTD protocol was 900 pulses of 1Hz stimulation as described (Dudek and Bear, 1992). Recordings were performed with axopatch 200 (Axon Instruments, Inc). Signals were filtered at 2kHz, digitized at 5kHz and analyzed with programs written in AXOBASIC. The initial slopes of field excitatory postsynaptic potentials (f-EPSPs) and the peak amplitudes of EPSCs were measured for field potential and whole-cell recordings, respectively. CNQX was from Cambridge Research Biochemicals and D(-)-AP5 was from Research Biochemicals Inc.

Time course and magnitude of the NMDA receptor components, and the ratios of non-NMDA to NMDA receptor component of synaptic currents were obtained from whole-cell patch clamp recording experiments in which EPSCs were monitored before and after application of 10μM CNQX from the same cell at holding potential of -50mV in 1.3mM Mg2+. Charge carried by NMDA receptor component measured in the presence of CNQX was compared to the charge of synaptic current before CNQX application that represents both non-NMDA and

NMDA components. The ratios of NMDA component to the sum of NMDA and non-NMDA components did not differ between the wild type and mutant neurons: for 10 wild type neurons (n=10 slices, 4 mice) this ratio was 40 ± 4% (±SEM), and for 10 mutant neurons (n=10 slices, 4 mice) it was 36 ± 3% (±SEM). Consequently the ratios of non-NMDA to NMDA components of synaptic currents are also indistinguishable between the mutant and the wild type cells.

Not all the data used to prepare cumulative histograms shown in Figures 8A and 10B were included in the averaged traces displayed in Figures 7 and 10B since a few of the data files were not compatible with our analysis program that was used for averaging data. Cumulative histograms of responses obtained only from those data used for the averaged trace are indistinguishable from the cumulative histograms presented in the manuscript (data not shown).

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# FIGURES

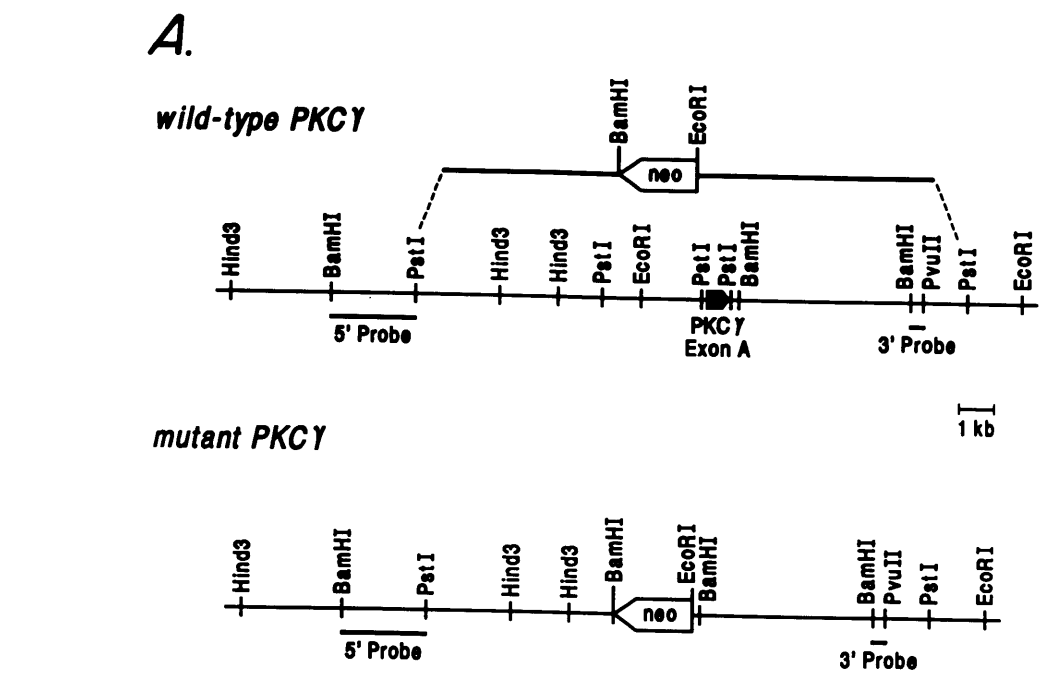
**Figure 1.** Production and analysis of PKCγ mutant mice.

(A) The PKCγ locus and targeting construct. A region of the PKCγ gene is shown that includes exon A, encoding the nucleotide-binding domain (for review see Nishizuka, 1988). A 2.5 kb fragment from the PKCγ gene including exon A was deleted and replaced with a neo gene. PKCγ 5' and 3' probes used for screening of ES cell clones and mice are indicated.

(B) Southern blot analysis of representative ES cell tail biopsies. DNA was digested with BamHI (top) or EcoRI (bottom) and hybridized with the 5' or 3' probes, respectively. Wild type and targeted alleles are indicated. Two mice in the litter are homozygous for the mutation.

(C) Northern blot analysis of PKCγ expression in wild type, heterozygote, and PKCγ mutant mice. PKCγ exon A RNA transcripts are absent in PKCγ mutant mice. Human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) is a housekeeping gene that cross-hybridizes with the mouse gene and serves to control for RNA quantity.

(D) Western blot analysis of PKCγ and PKCb expression in wild type, heterozygote, and PKCγ mutant mice. PKCγ immunoreactivity is absent in brain extracts from PKCγ mutant mice, whereas PKCb immunoreactivity does not appear to be altered in PKCγ mutant mice.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BamHI
BamHI
PvuII
PstI
EcoRI
5' Probe
PKCγ
Exon A
3' Probe
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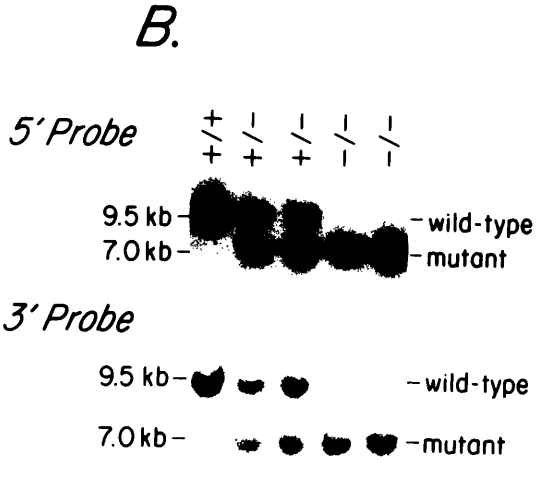
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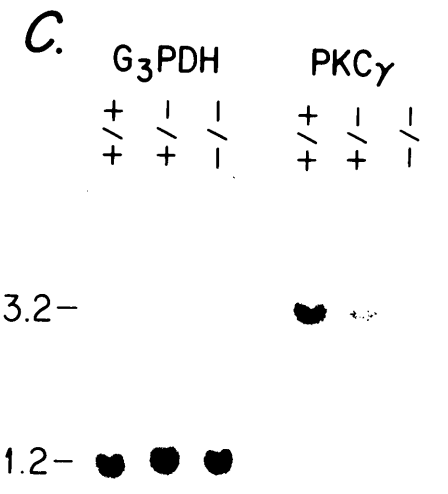
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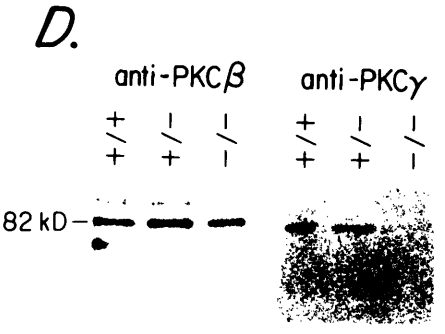
D.
anti-PKCβ
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+/+ +/- -/-
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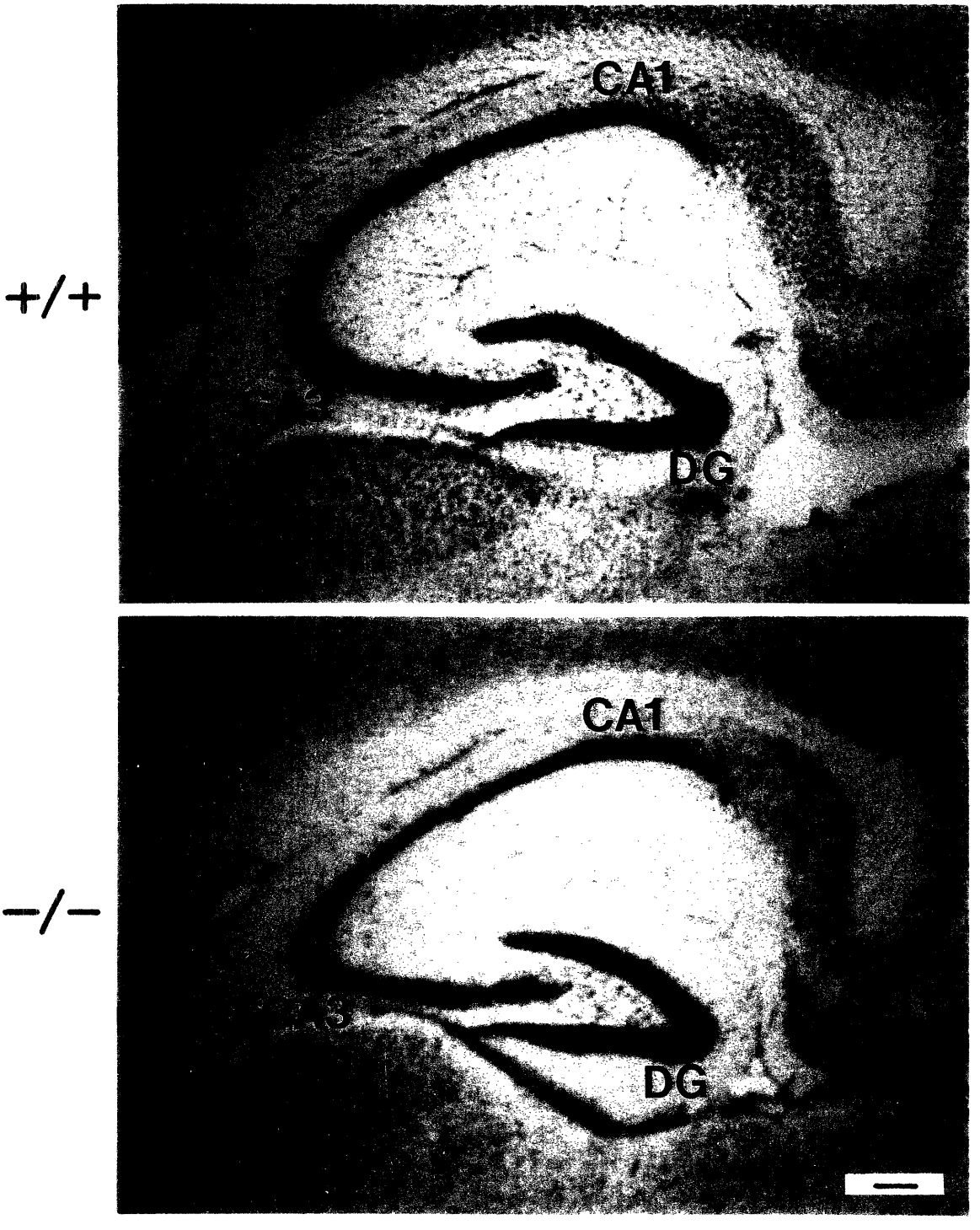
**Figure 2.** Transverse sections of hippocampus from wild-type and PKCγ mutant mice stained with cresyl violet. Cell body layers of CA1, CA3 and dentate gyrus (DG) are indicated. Scale bar, 200 υ.


+/+
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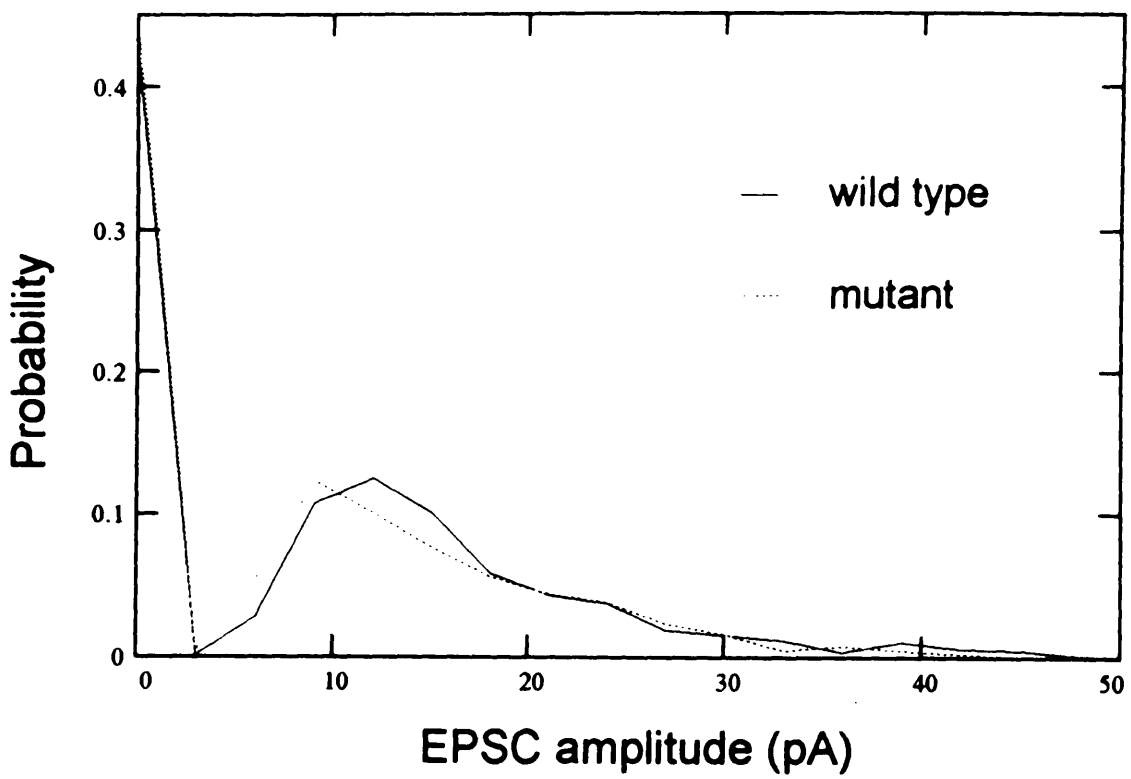
CA1
DG

**Figure 3.** Amplitude histogram of responses to minimal stimulation. Responses were recorded in wild type (solid line) and mutant slices (dashed line) in whole-cell patch clamp configuration. Stimulus intensity was adjusted such that failure rate of responses elicited was ~40%. The histogram shows the probability of observing a response as a function of response size (epsc amplitude). Bin size is 3pA. Distributions of evoked responses are virtually indistinguishable between the wild type (864 trials; n=7 cells, 7 slices from 5 mice) and the mutant (894 trials; n=7 cells, 7 slices from 5 mice) cells. This indicates that mutant slices display normal postsynaptic responsiveness. Two wild type and two mutant example traces are displayed.


Probability
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
wild type
mutant
EPSC amplitude (pA)

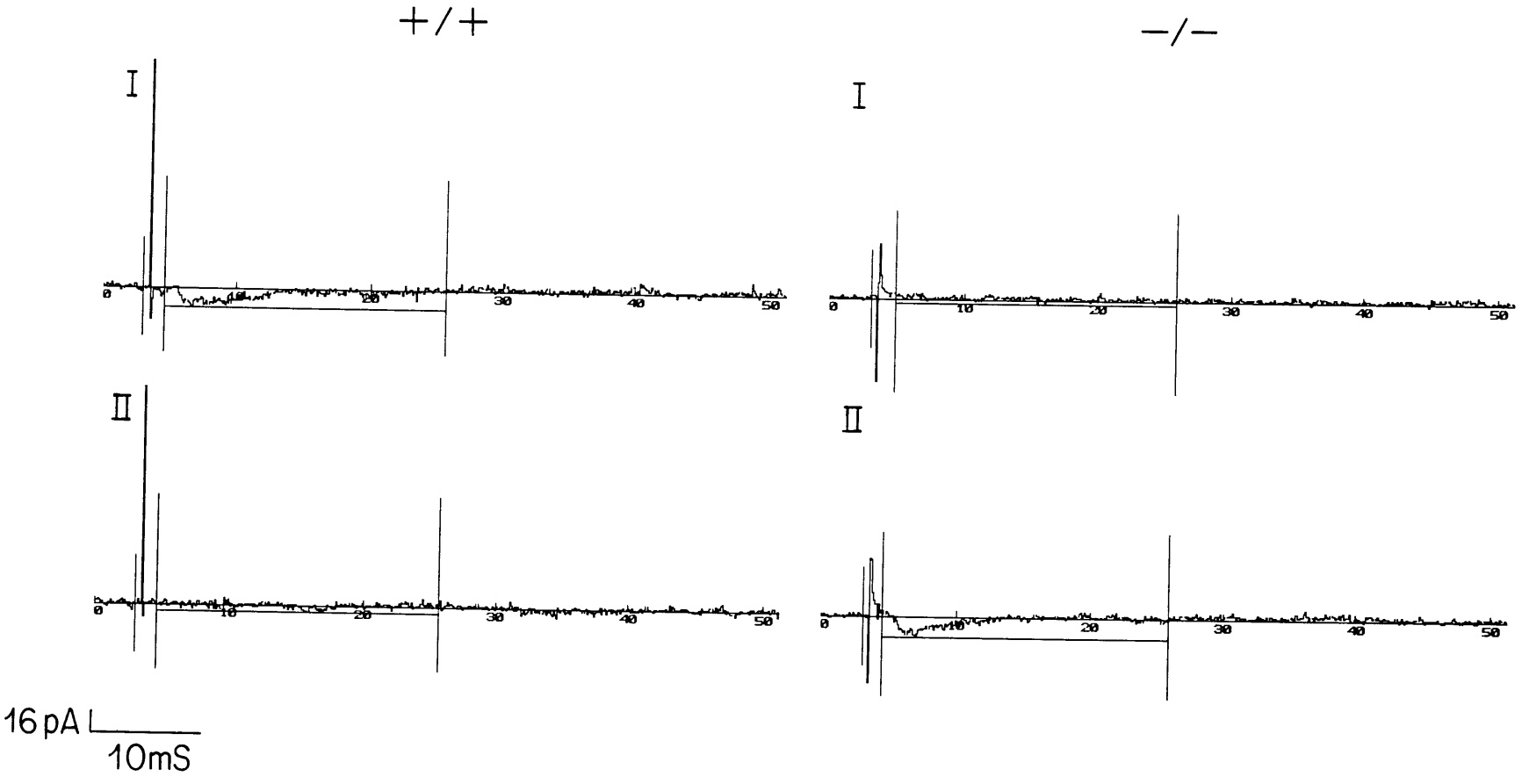
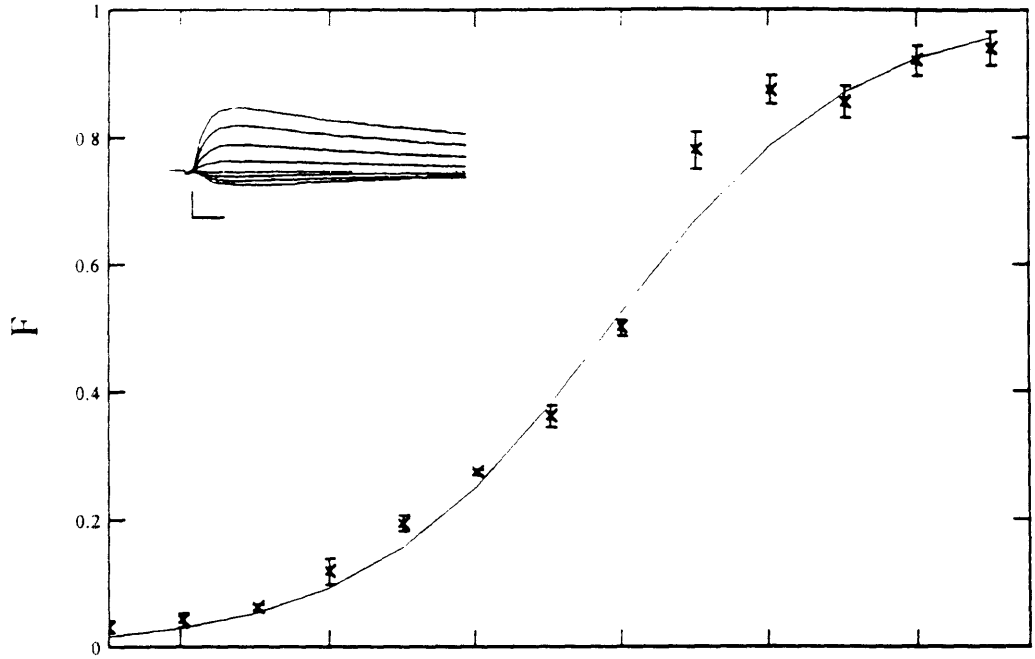

+/+
−/−
I
II
I
II
0
10
20
30
40
50
16 pA
10mS

**Figure 4.** Voltage dependence of NMDA receptors.The perfusate contained 10mM CNQX to block non-NMDA receptor component of synaptic currents. Fraction (F) of the maximum conductance through the NMDA receptor channels is plotted as a function of holding membrane potentials. Data from 4 wild type cells (top; 4 slices from 2 mice) and 5 mutant cells (bottom; 5 slices from 3 mice) are shown. Error bars display standard error of the mean. Insets show sample traces of synaptic currents at various holding potentials (mV): from top to bottom, +50, +40, +30, +20. -70, -50, 0 and -20 for the wild type cell and +40, +30, +20, -60, -40, 0 and -20 for the mutant cell. Calibration bars are 10 msec and 100pA (wild type trace) or 200pA (mutant trace). The smooth curves were fitted with the following equation:

$$g(V) = 1/[1 + \exp( - 0.062V)(C/3.57)]$$

where $\gamma$ is conductance in picosiemens, V is membrane potential in millivolts, and C is the extracellular magnesium concentration in millimoles / liter (Jahr and Stevens, 1990).

Wild type




Mutant

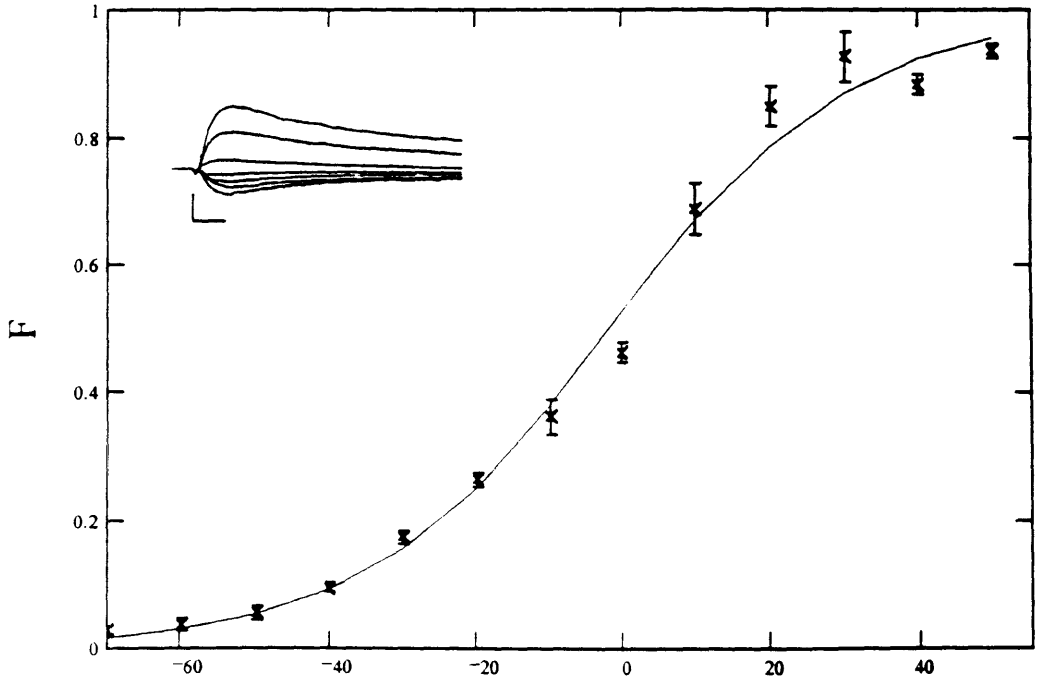


**Figure 5.** Paired-pulse facilitation in wild type and mutant slices. Field potential responses to pairs of stimuli were monitored in wild type (solid line; 11 slices from 2 mice) and mutant (dashed line; 12 slices from 4 mice) animals. The second response, expressed as % response to the first pulse, is plotted as function of interpulse intervals. The error bars display the standard error of the mean. Ca2+/Mg2+ ratio of the perfusing medium was optimized for evoking paired pulse facilitation and was decreased to 1mM Ca2+, 2.4mM Mg2+ (from 2.5mM Ca2+, 1.3mM Mg2+).

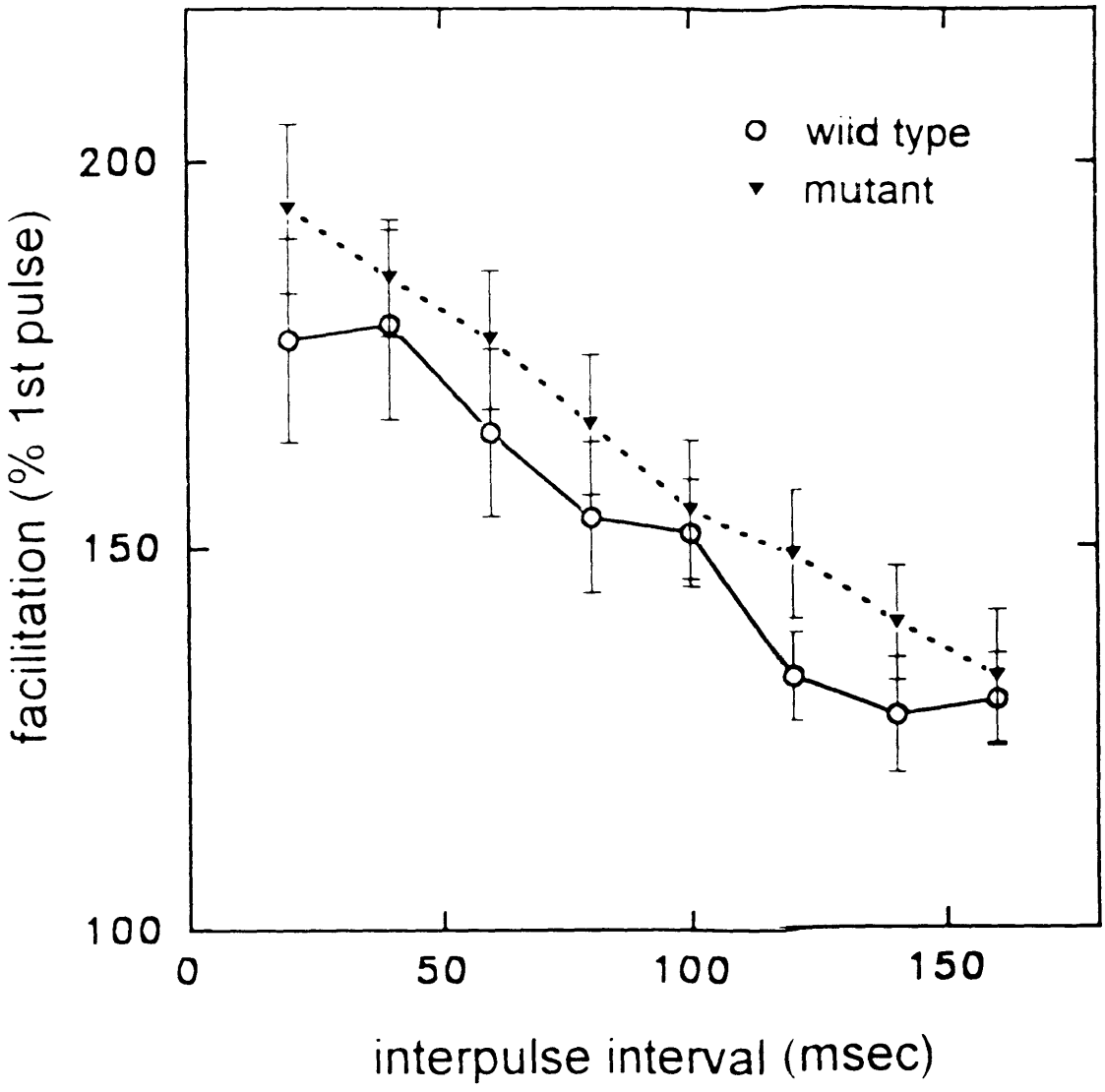

wiid type
mutant
200
150
100
0
50
100
150
facilitation (% 1st pulse)
interpulse interval (msec)

**Figure 6.** Cumulative histogram of LTD. Low frequency stimulation consisting of 900 pulses at 1Hz elicited LTD in both wild type and mutant slices. Cumulative probability is shown as a function of mean LTD responses measured 20~30 min after the low frequency stimulation. Response size is expressed as % baseline response prior to low frequency stimulation. Field potential recordings from 26 wild type (solid line; 10 mice) and 29 mutant slices (dotted line; 9 mice) are presented. Standard deviations for wild type and mutant slices were 16.7 and 18.4, respectively. Mean LTD responses were 90 ± 3% (wild type) and 87 ± 3% (mutant).

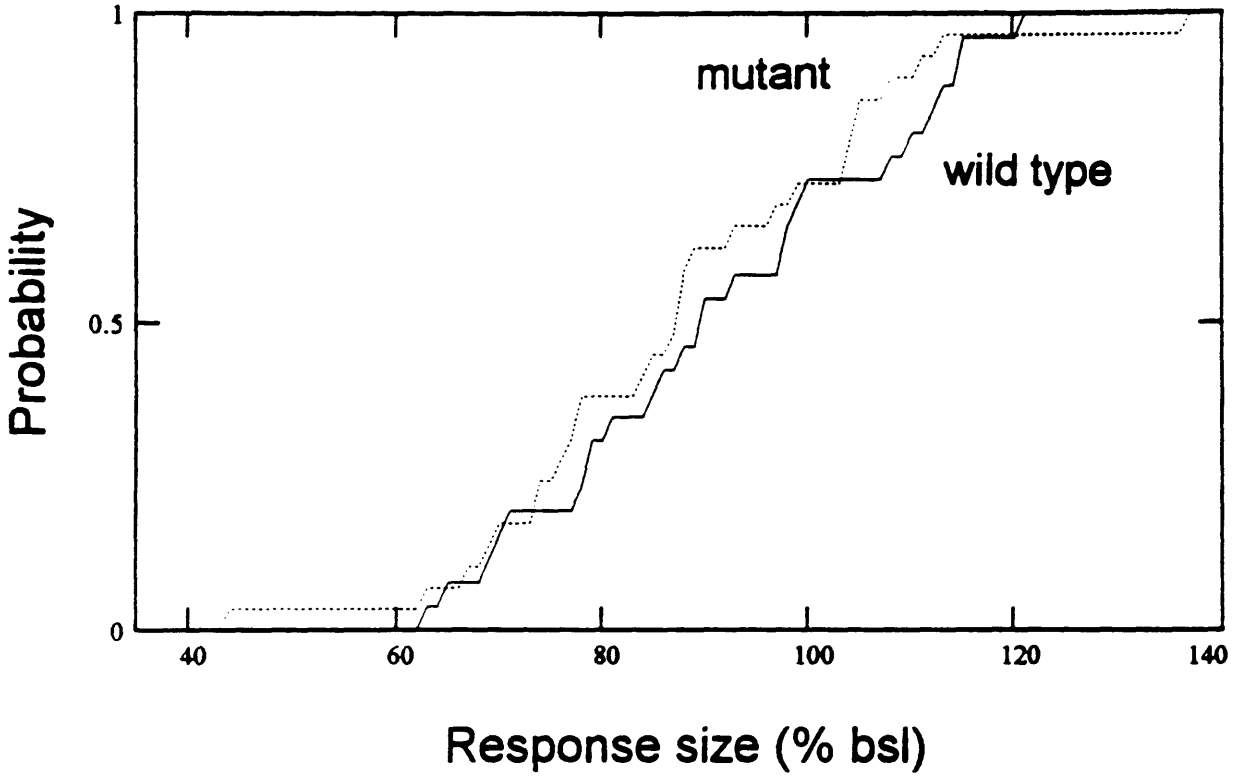

1
0.5
0
Probability
mutant
wild type
40
60
80
100
120
140
Response size (% bsl)

**Figure 7.** LTP is abnormal in mutant slices. Summary of field potential recordings from 15 wild type (A; n=6 mice) and 13 mutant (B; n=5 mice) slices are shown. Initial slope of field excitatory postsynaptic potentials (f-EPSPs) are expressed as percentage of the mean baseline f-EPSP slope before tetanic stimulation (arrow). The error bars display the standard error of the mean. Tetanus to evoke LTP consisted of 5 trains of 100Hz stimulation, each lasting 200msec at an intertrain interval of 10sec. Testing stimuli were given every 20sec.

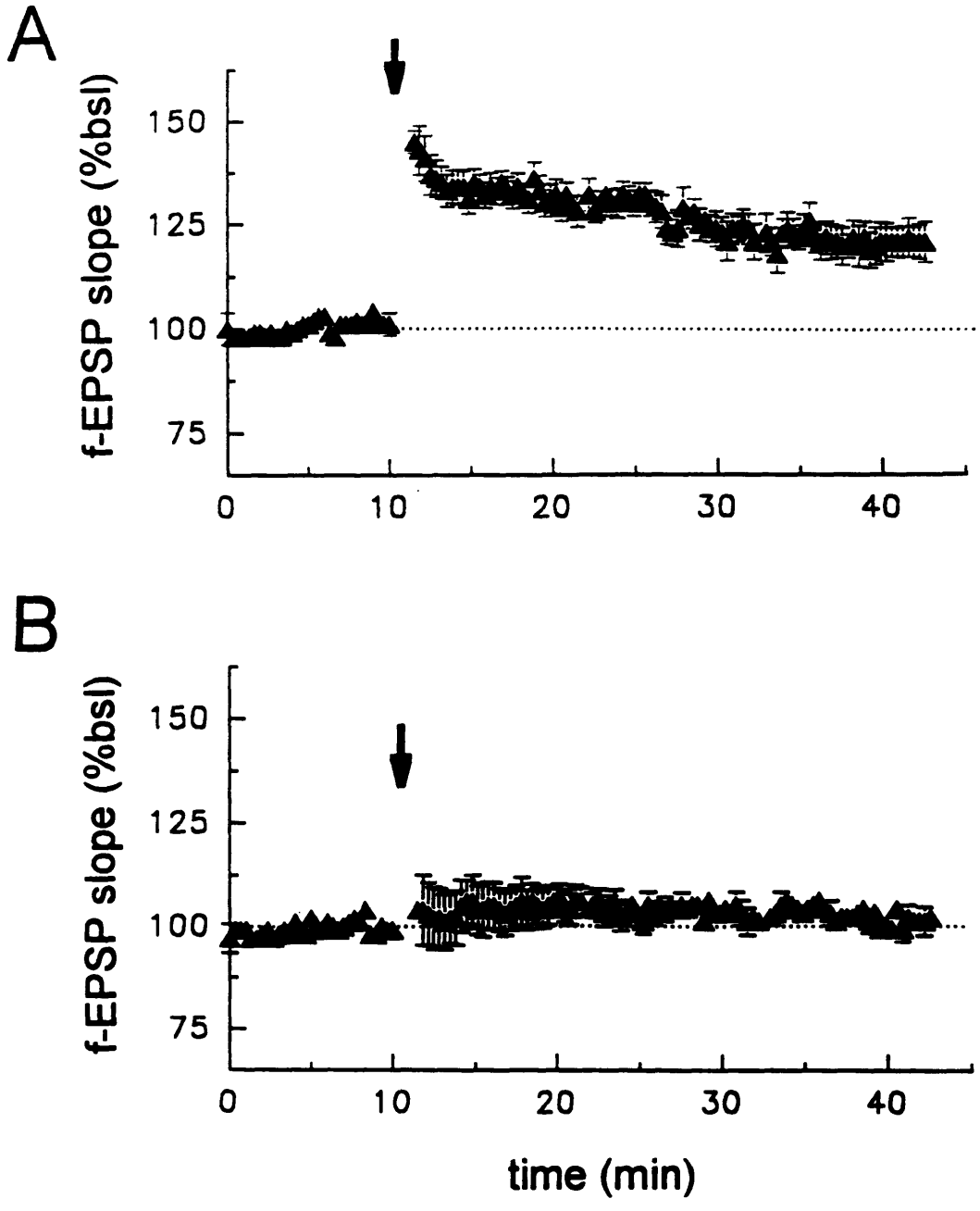

A
f-EPSP slope (%bsl)
150
125
100
75
0
10
20
30
40
B
f-EPSP slope (%bsl)
150
125
100
75
0
10
20
30
40
time (min)

**Figure 8.** Cumulative histograms of LTP. Cumulative probability is shown as a function of mean LTP response size measured between 20~30 min after tetanus. Response size is expressed as % of the baseline response prior to tetanus. (A) Field potential recordings from 17 wild type (solid line; 6 mice) and 14 mutant slices (dotted line; 6 mice). Mean LTP responses are 124 ± 5% and 101 ± 2% for wild type and mutant slices respectively. Standard deviations of probability distributions are 21% (wild type ) and 8% (mutant). (B) Whole-cell recordings from 7 wild type (solid line; 3 mice) and 12 mutant slices (dotted line; 4 mice) are shown. Mean LTP responses are 141 ± 13% for wild type and 93 ± 4% for mutant slices. Standard deviations of probability distributions are 31% and 14% for wild type and mutant slices, respectively.

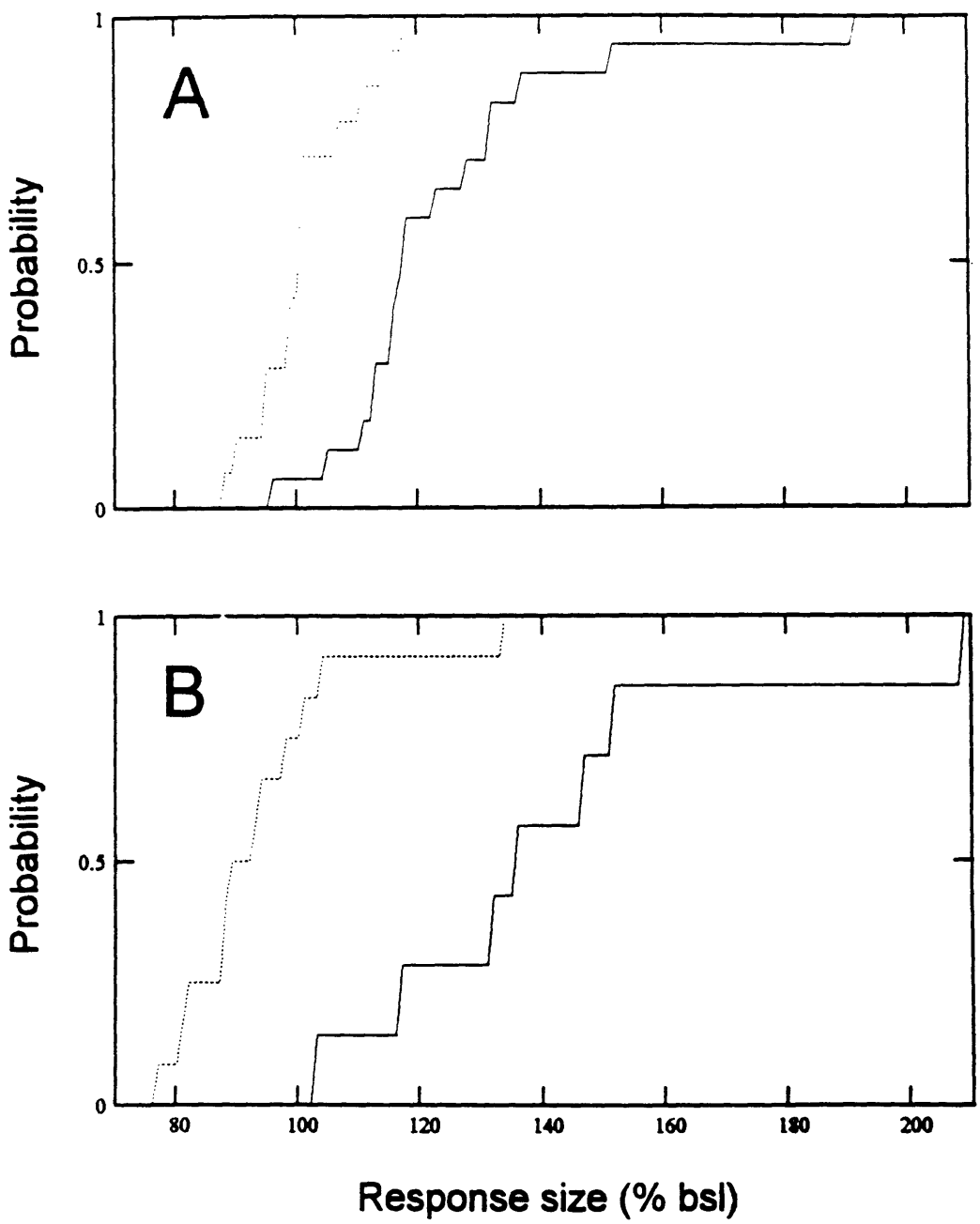

A
B
Probability
Probability
1
0.5
0
1
0.5
0
80
100
120
140
160
180
200
Response size (% bsl)

**Figure 9**. LTP is either absent or greatly diminished in mutant slices. (A) Comparison of cumulative probability distributions of LTP amplitude between untreated mutant slices (solid line; n=14 from 6 mice) and mutant slices treated with 50mM AP5 (dotted line; n=10 from 2 mice). Mean responses are 101 ± 2% and 95 ± 5% for mutant slices without or with AP5 treatment respectively. Cumulative distribution of LTP responses in mutant slices is indistinguishable from the distribution of responses measured in the presence of LTP inhibitor. (B) Cumulative distributions of 9 wild type (solid line; 2 mice) and 10 mutant slices (dotted line; 2 mice) that have been treated with 50mM AP5. Mean responses are 96 ± 3% (wild type+AP5) and 95 ± 5% (mutant+AP5). When LTP induction is disabled both mutant and wild type slices display indistinguishable cumulative probability distributions.

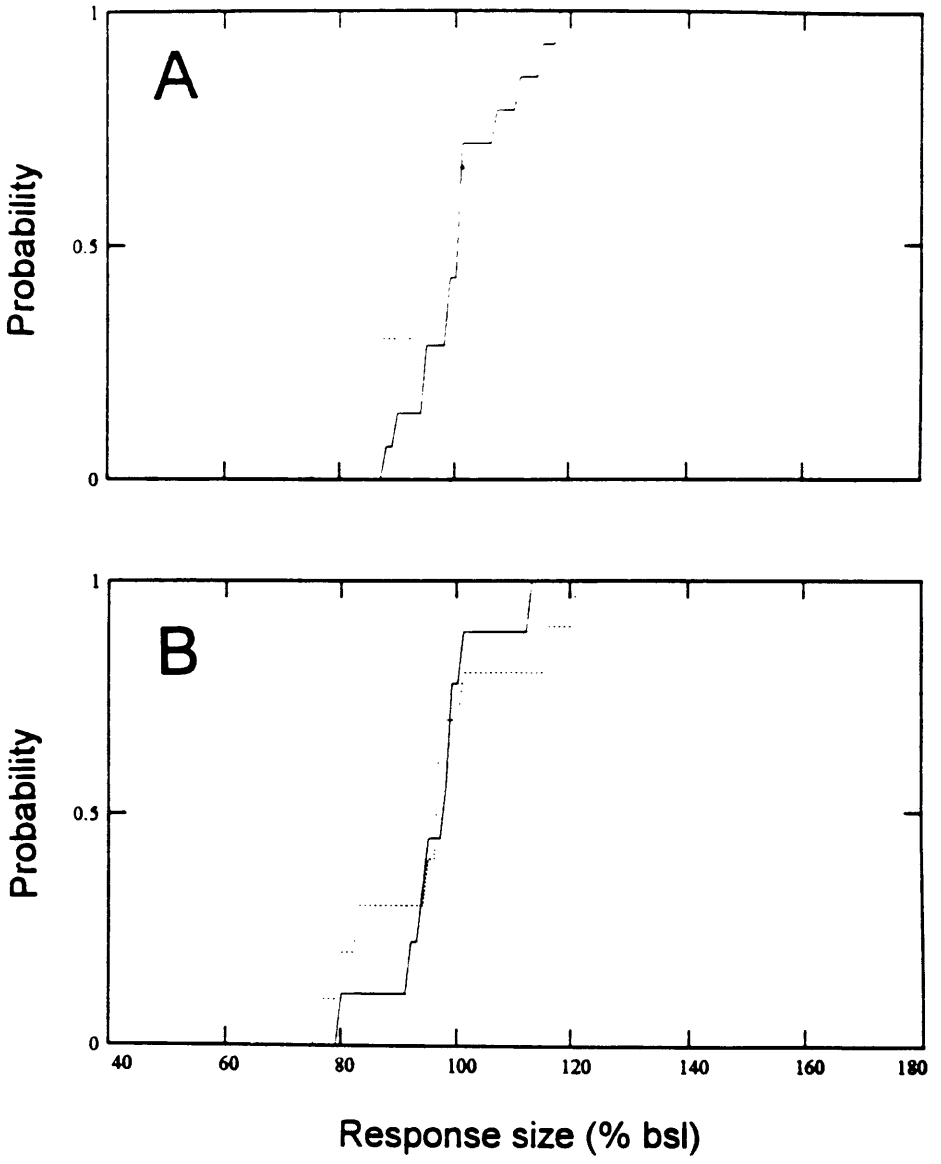
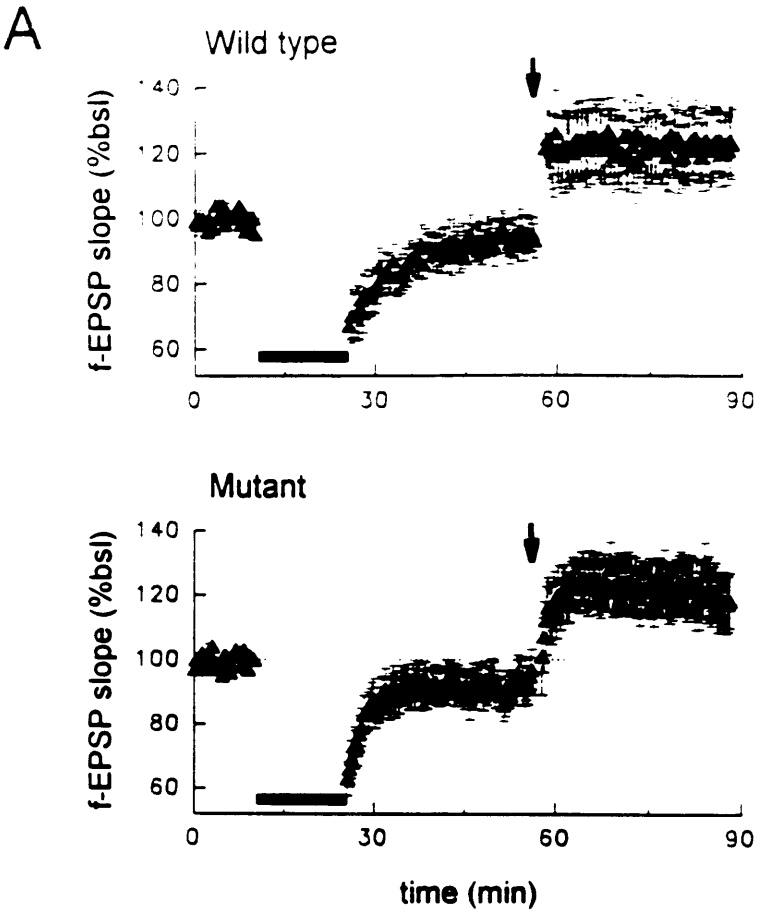
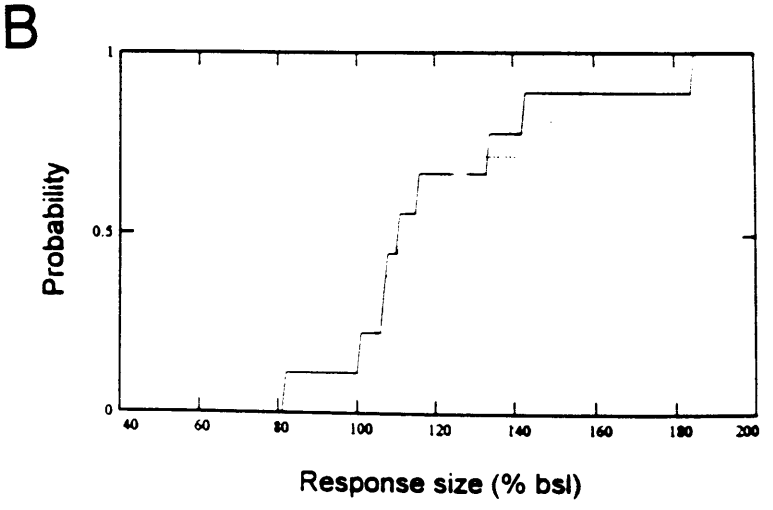
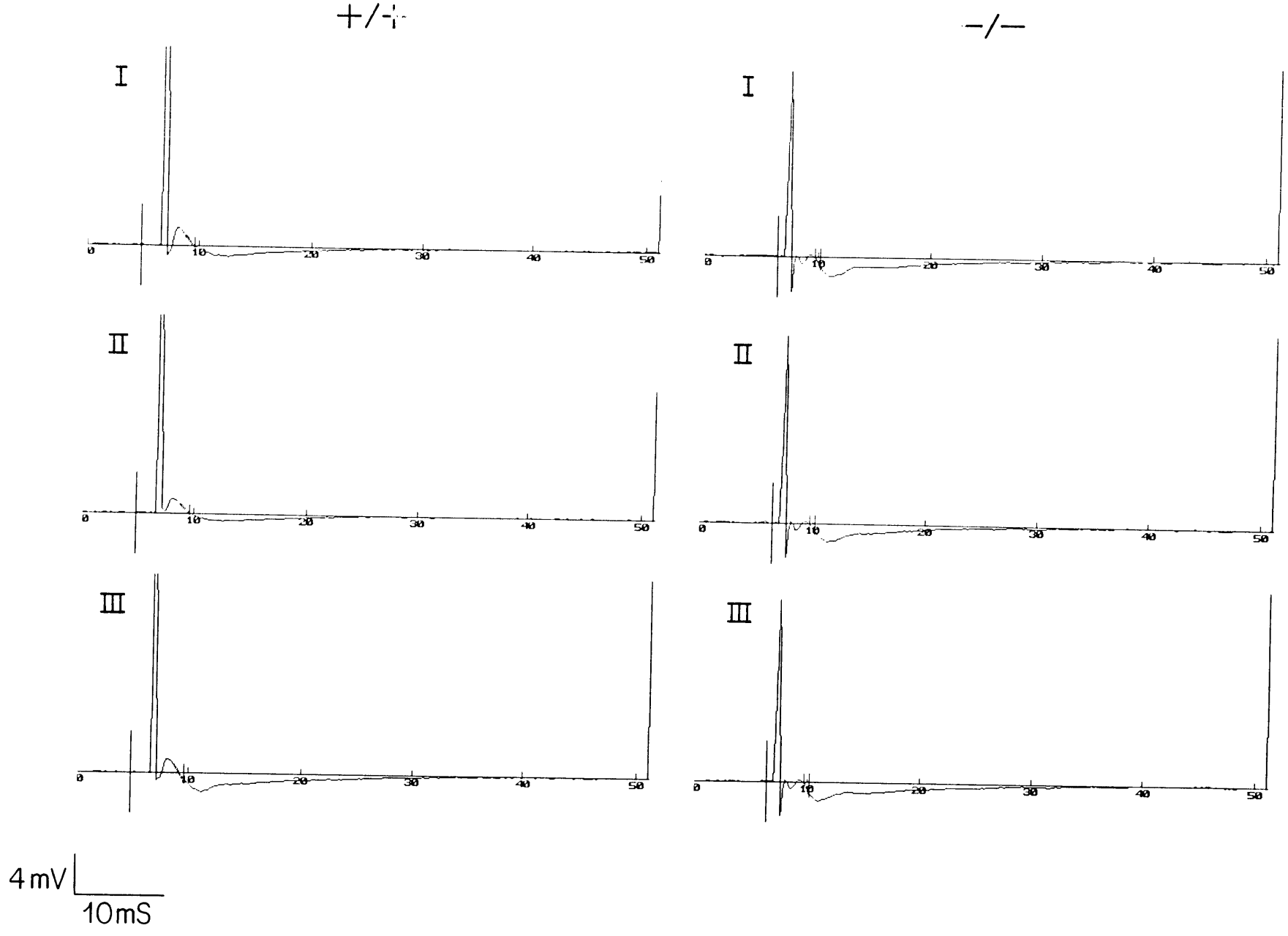

A
B
Probability
Probability
1
0.5
0
1
0.5
0
40
60
80
100
120
140
160
180
Response size (% bsl)

**Figure 10.** LTP can be elicited in mutant slices after the LTD protocol. (A) Summary of field potential recordings from 8 wild type (5 mice) and 15 mutant slices (9 mice) where LTD protocol (solid bar) was followed by a tetanus to evoke LTP (arrow). Responses are expressed as % of the baseline response (f-EPSP slope) prior to low frequency stimulation. The error bars display the standard error of the mean. (B) Cumulative probability distributions of LTP responses from 9 wild type (solid line; 5 mice) and 21 mutant slices (dotted line; 9 mice) where LTD protocol was given prior to tetanus to elicit LTP. Average LTP responses measured between 20~30 min after tetanus is expressed as % of the average baseline response obtained before the application of LTD protocol. Wild type and mutant slices displayed mean LTP responses of 120 ± 10% and 113 ± 8% respectively. Example wild type and mutant traces recorded prior to low frequency stimulation (I), after low frequency stimulation (II), and after high frequency stimulation (III) are shown.


A
Wild type
f-EPSP slope (%bsl)
140
120
100
80
60
0
30
60
90
Mutant
f-EPSP slope (%bsl)
140
120
100
80
60
0
30
60
90
time (min)


B
Probability
1
0.5
0
40
60
80
100
120
140
160
180
200
Response size (% bsl)


+/+
-/-
I
II
III
I
II
III
0
10
20
30
40
50
4mV
10mS

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# CHAPTER 3

# PKCγ Mutant Mice Exhibit Mild Deficits in Spatial and Contextual Learning

**Asa Abeliovich*, Richard Paylor+, Chong Chen*#, Jeansok J. Kim%, Jeanne M. Wehner+, and Susumu Tonegawa*#**

*Center for Cancer Research and Department of Biology
Massachusetts Institute of Technology
Cambridge, MA 02139

#Howard Hughes Medical Institute at the Center for Cancer Research and Department of Biology
Massachusetts Institute of Technology
Cambridge, MA 02139

+Institute for Behavioral Genetics and School of Pharmacy
University of Colorado
Boulder, CO 80309

%Neuroscience Program
University of Southern California
Los Angeles, CA 90089-2520

**Running Title: PKCγ mutant behavior**

## SUMMARY

**We are undertaking a genetic approach to investigate the role which synaptic modulation in the mammalian CNS plays in learning and memory and to identify relevant molecular components. We have generated mice deficient in the γ isoform of protein kinase C (PKCγ), an enzyme that has previously been implicated in both long-term potentiation (LTP) and learning and memory. These mice have a modified LTP of synaptic transmission in the hippocampus (Abeliovich et al., 1993). We demonstrate that PKCγ mutant mice can learn to carry out hippocampus-dependent tasks, although mild deficits are evident. Thus, hippocampal CA1 LTP induced by the conventional tetanic stimulation is not essential for the mice to exhibit spatial and contextual learning. Furthermore, the modification of hippocampal synaptic plasticity correlates with the learning deficits we observe.**

## INTRODUCTION

Donald Hebb theorized that the physiological basis of learning and memory involves the long-term strengthening of synapses among neurons that are coincidentally active (Hebb, 1949). Subsequently, a form of synaptic strengthening was identified, termed long-term potentiation (LTP), that exhibits the properties Hebb initially postulated (Bliss and Gardner-Medwin, 1973; Bliss

and Lomo, 1973): coincident presynaptic and postsynaptic neuronal activity is required for its induction; the modulation is specific for the activated synapse; and it is long-lasting. A second form of synaptic change or plasticity, termed long-term depression (LTD), has recently been defined in the hippocampus (Dudek and Bear, 1992; Mulkey and Malenka, 1992) that also conforms to these rules, except it involves the weakening of synaptic connections. Both forms of synaptic plasticity are present in the hippocampus, a structure implicated in learning and memory by lesion experiments (Squire, 1987).

Hippocampus lesions have served to define two classes of associative learning: a complex class of learning termed declarative or configural that is sensitive to hippocampal lesions; and a simple class of learning termed procedural that is insensitive to hippocampal lesions (Squire, 1987; Eichenbaum et al., 1991; Eichenbaum et al., 1992; Sutherland and Rudy, 1989; Jarrard, 1993; Hirsh, 1974; Squire, 1992; O'Keefe and Nadel, 1978). Hippocampus-dependent learning tasks commonly involve associations among multiple sets of cues, whereas hippocampus-independent tasks commonly involve a simple association. In rodents, configural learning is often evaluated in the hidden-platform Morris water maze navigation task. This task involves the association of multiple spatial cues, a process termed spatial learning that is a subclass of configural learning. More recently, other configural tasks such as context-dependent fear conditioning (Kim and Fanselow, 1992; Phillips and LeDoux, 1992) have been identified in rodents.

It has been proposed that LTP might serve as a mechanism by which synapses are strengthened in the course of hippocampal-dependent learning and memory (Bliss and Lomo, 1973). In order to test this hypothesis, Morris and

others examined the spatial learning capability of rats whose hippocampal LTP had been blocked by AP5, an antagonist for glutamate receptors of the N-methyl-D-aspartate (NMDA) class (Morris et al., 1986; Morris et al., 1991). Since these rats were defective in both LTP and spatial learning, at least under certain conditions, these data suggest that LTP is the cellular mechanism for this type of learning. More recently, a genetic approach was applied to examine further the relationship between hippocampal LTP and spatial learning (Silva et al., 1992a; Silva et al., 1992b). Mice genetically deficient in the α isoform of calcium, calmodulin dependent protein kinase II (αCaMKII) were generated using the gene targeting technique. These mutant mice, although normal in ordinary synaptic transmission, are markedly deficient in hippocampal LTP and are severely impaired in spatial learning. Thus, the αCaMKII mutant study supports the hypothesis that LTP is the cellular mechanism for spatial learning. A similar conclusion (Grant et al., 1992) was drawn from the analysis of mice deficient in *fyn*, the gene encoding the non-receptor tyrosine kinase Fyn.

While the cumulative evidence cited suggests a critical role for LTP in certain types of learning and memory, several issues remain unresolved. For instance, blocking NMDA receptors disrupts LTP, but also disrupts synaptic function and thus potentially interferes with the in vivo computational ability of hippocampal circuits (see Bekkers and Stevens, 1990). Therefore, perhaps the failure of learning results not from the deficit in LTP but simply from incorrect operation of hippocampal circuits. In the *fyn* mutants, a clear defect was found in the arrangement of the granule cells and the pyramidal cells of the CA3 region. Thus, a developmental abnormality, rather than the LTP blunting, could be the cause of the observed impairment in spatial learning. Furthermore, in the three studies cited, LTD was neither examined nor considered as a candidate cellular

mechanism for learning and memory. It is now known that the NMDA receptor antagonist AP5 blocks the induction of hippocampal LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992) as well as LTP, and furthermore hippocampal LTD is impaired in αCaMKII mutant mice (C. F. Stevens, S.T., and Y. Wang, unpublished observations).

Thus, it is necessary to examine further the relationship between LTP (or LTD) and learning and memory. To this end, we have generated a strain of mouse mutant with a deletion in the gene encoding the γ isoform of protein kinase C (PKC). We chose to mutate PKC because PKC inhibitors have been shown to block LTP (Malinow et al., 1988; Malinow et al., 1989), indicating that PKC activity is required for LTP. Furthermore, several experiments have correlated hippocampal PKC with performance in learning tasks (Bank et al., 1989; Olds and Alkon, 1991), although no causal role for PKC has been established in learning and memory. For example, classical conditioning of the nictitating membrane response in rabbits has been correlated with increased phorbol ester binding in the hippocampus (Bank et al., 1988), and spatial learning performance of rats (Olds et al., 1990; Paylor et al., 1992) and mice (Wehner et al., 1990; Fordyce and Wehner, 1993) in the hidden-platform Morris water maze task has been correlated with hippocampal PKC activity.

PKC is composed of a family of at least ten isoforms encoded by at least nine genes (Nishizuka, 1988). In none of the LTP or learning experiments cited above has the specific functional role of the various isoforms been determined. In the present study, we chose to focus on the γ isoform because it is specific for neurons in the central nervous system (CNS) and is expressed postnatally (Hashimoto et al., 1988), in contrast to several other isoforms. Furthermore, an

increase in the measured γ isoform in the hippocampus has been associated with spatial learning (Van der Zee et al., 1993), although the involvement of other isoforms cannot be excluded. An accompanying paper (Abeliovich et al., 1993) shows that LTP in the CA1 hippocampal region of PKCγ mutant mice is abnormal in that it can rarely be induced after conventional high frequency stimulation (tetanus), although apparently normal LTP can be observed if the tetanus is preceded by a low frequency (1 Hz) stimulation. Abeliovich et al. (1993) also show that the CA1 hippocampal region of mutant mice is normal in the induction of LTD as well as in ordinary synaptic transmission. In this paper, we analyze PKCγ mutant mice in two different learning tasks both of which require an intact hippocampus in rodents but which differ in their performance requirements and allow for the assessment of nonspecific behavioral impairments.

## RESULTS

PKCγ mutant mice are viable and display normal grooming, feeding, circadian activity, and mating behaviors. However, PKCγ mutant mice have an abnormal gait. For example, when walking on a floor made of round steel rods 1 cm apart, PKCγ mutant mice often misplace their limbs between rods. In addition, mutant mice are more prone to falling off of an inclined pole (3 cm diameter) than wild-type mice. The observed coordination deficits appear to ameliorate with practice. This phenotype is typically observed in cerebellum-lesioned animals (Flourens, 1824) and, although mild, suggests a role for PKCγ in cerebellar physiology, consistent with its high level of expression in cerebellar Purkinje cells (Nishizuka, 1988).

LTP is modified in PKCγ mutant mice in that it can rarely be induced by conventional stimulation (Abeliovich et al., 1993). To test the correlation between hippocampal LTP and learning and memory, we subjected PKCγ mutant mice to two learning and memory tasks, the performance of which is reported to require the hippocampus in rodents: a hidden-platform water maze task that tests spatial learning, and context-dependent fear conditioning, a task that tests contextual learning. Additionally, we investigated the performance of mutant mice in two tasks that, in rodents, do not require the hippocampus: a visible-platform water maze task (Morris et al., 1982; Sutherland et al., 1982), and tone-dependent fear conditioning (Kim and Fanselow, 1992; Phillips and LeDoux, 1992).

**Morris Water Maze**

The Morris water maze (Morris, 1981) consists of a circular pool filled with opaque water and containing an escape platform submerged approximately 1 cm below the surface of the water. In the hidden platform version of the Morris water maze task, mice are placed in the pool at one of 4 start sites, and the platform location is kept constant throughout training. In order to escape the water, mice must learn to navigate to the hidden platform by mapping its position relative to visual cues outside of the pool, a process defined as spatial learning. In the visible-platform Morris water maze task, a cylindrical landmark is placed on the escape platform, indicating its position. Mice are placed in the pool at one of four start sites but, unlike the hidden-platform, the "visible" platform is relocated to new quadrants of the pool between trials. Therefore, mice must learn to associate the landmark with the location of the platform, and spatial information is irrelevant. In rodents, disruption of NMDA receptor function appears to impair LTP induction as well as performance on the hidden-platform

Morris water maze task (Morris et al., 1986), whereas performance on the visible-platform task is unimpaired (Morris et al., 1991). Because the two tasks are similar in terms of motivation and the requirement for swimming ability, the visible-platform task serves as an important control for these factors.

**Visible-platform Morris water maze Task**

PKCγ mutant mice were tested in the visible-platform version of the Morris water maze, a non-spatial learning task, following training on the hidden-platform task. The performance of PKCγ mutant mice (n=8) was not significantly different (p=.122) from that of wild-type mice (n=7), although mutant mice tended to perform somewhat more poorly than wild-type mice initially (Figure 1). In the course of training, mutant mice did reach the wild-type level of performance, demonstrating that mutant mice can learn this task, and suggesting that the initial impairment displayed by mutant mice does not prevent them from learning. Similar results were observed when the mice were not trained on the hidden-platform task prior to visible-platform training (data not shown).

**Hidden-platform Morris water maze task**

Spatial learning was tested in the hidden-platform version of the Morris water maze task. Both mutant (n=12) and wild-type (n=9) mice displayed significant improvement over the 9 blocks of training ($p<.0001$), and the two groups did not differ significantly (p=.598) (Figure 2A). Mice can improve their performance in the hidden-platform task by adopting a learning strategy other than spatial learning. For instance, the mice may learn that the platform is located a certain distance away from the edge of the pool. This strategy is not as precise as the spatial learning strategy, but it nevertheless enables mice to find the platform more quickly than a random search does.

In order to confirm that the PKCγ mutant mice used a spatial learning strategy, we subjected the trained mice to a probe test in which the platform was removed and the mice were allowed to search the pool for 60 seconds. Both mutant ($p<.0001$) and wild-type ($p<.0001$) mice selectively searched the quadrant in which the platform had been located during the training versus all other quadrants (Figure 2B). Furthermore, both mutant ($p<.0001$) and wild-type ($p<.0001$) mice crossed the exact location at which the platform had been located during training more frequently than any of the corresponding locations in the other quadrants (Figure 2C). However, in this platform site-crossing test, mutant mice did cross the correct site less often than wild-type mice ($p<.05$), indicating that mutants harbor a moderate deficit in spatial learning.

We also subjected the trained mice to another test, the "random" platform test, to evaluate again the search strategy of PKCγ mutant mice. In this test, the platform was placed at either its original training location or at a corresponding location in any one of the other three quadrants, and the time required to locate the platform was compared for the training versus new locations. Both mutant and wild-type mice were able to locate the hidden platform at the original location significantly more rapidly than at new locations ($p<.0001$) and the two groups of mice did not differ significantly in the time required to locate either platform ($p=.985$) (Figure 2D). Thus, the results of the "random" platform test corroborated those of the probe test and demonstrated that the PKCγ mutant mice can acquire spatial learning, although a mild deficit is detectable by the platform crossing test. Furthermore, when mutant mice were given more intensive training (massed-training; see Methods and data not shown) no

significant genotype differences were observed on any hidden-platform task measures assessed.

Most of the behavioral analyses were performed in the 129/Ola X C57BL/6 (B6) genetic background because preliminary experiments indicated that this genetic background performed best in the hidden-platform Morris water maze task. We also carried out an analysis of spatial learning with the PKCγ mutation in another genetic background, 129/Ola X Balb/C (C), to investigate the role of genetic background with respect to the PKCγ mutation. This second genetic background was chosen in order to allow for a comparison with the performance of αCaMKII mutant mice. Learning was somewhat blunted in the C genetic background relative to the B genetic background for both wild-type (n=8) and PKCγ mutant mice (n=7) (Figure 3). However, both wild-type and mutant mice of the C genetic background did show learning during acquisition (Figure 3A; $p<.001$), in the probe test (Figure 3B; $p<.001$), and in the random platform test (Figure 3C; $p<.001$). Furthermore, mutant and wild-type mice of the C genetic background did not differ significantly (all $p> .5$). This result confirms that PKCγ mutant mice display spatial learning in the hidden-platform Morris water maze task, and indicates that the difference in spatial learning performance between PKCγ mutant mice and αCaMKII mutant mice is not a consequence of the genetic background.

PKCγ mutant mice display spatial learning in the hidden-platform Morris water maze task, although mutants are partially impaired relative to wild-type mice in the probe test. To investigate whether the PKCγ mutation is important in the retention of spatial memory, we tested wild-type and mutant mice in a probe one month subsequent to distributed training. Both mutant and wild-type mice

remembered the location of the platform (Figure 4). Mutant mice did not differ significantly from wild-type mice in terms of time spent in the target quadrant (Figure 4A; $p>.2$), but crossed the target platform site significantly less often than wild-type mice (Figure 4B; $p<.044$), reflecting their performance on the initial probe. Although both wild- type and mutant mice decreased their level of performance relative to the initial probe test, the decreases observed did not differ significantly. Thus, retention does not appear to be specifically affected by the PKCγ mutation.

**Context-dependent fear conditioning**

We tested PKCγ mutant mice in a second task, context-dependent fear conditioning (Kim and Fanselow, 1992), that has been shown to be dependent on the hippocampus and NMDA receptor function in rodents (Kim et al., 1991). In this task an initially neutral stimulus -- an experimental chamber -- is paired with an aversive, unconditioned stimulus -- an electric shock. A conditioned freezing response, characterized by an immobile, crouching posture, is observed upon subsequent presentation of the experimental chamber. Hippocampus lesions in rodents serve to define two forms of fear conditioning: classical conditioning of fear to nonspecific cues, such as the context of an experimental chamber, that is sensitive to hippocampal lesions; and conditioning to specific cues, such as a tone, that is insensitive to hippocampal lesions (Kim and Fanselow, 1992; Phillips and LeDoux, 1992).

In the conditioning phase of the experiment, mice were placed in a shocking chamber, and subsequently received 3 footshocks. One day later, mice were returned to the shocking chamber and monitored for freezing behavior. Wild-type ($n=14$) and PKCγ mutant ($n=13$) mice displayed comparable

freezing during the conditioning phase of the experiment (Figure 5A), demonstrating that the PKCγ mutant mice do not harbor a performance deficit in this task, such as an inability to freeze. The next day both mutants and wild-type mice displayed the conditioned freezing response to the training context. However, mutants froze significantly less than wild-type mice (Figure 5B; $p<.05$), indicating a moderate deficit in context-dependent fear conditioning.

Additionally, we analyzed context-dependent fear conditioning of wild-type and mutant mice of the 129/Ola X Balb/C (C) genetic background. We found that freezing performance in both the conditioning and the testing phases was considerably blunted for all mice of the C genetic background (data not shown) relative to the 129/Ola X C57BL/6 genetic background, similar to the performance of these strains in the hidden-platform Morris water maze task. We did not detect a deficit with PKCγ mutant mice relative to wild-type mice of the C genetic background (data not shown), but this is likely to be due to the generally poor performance of mice of the C genetic background, which reduces the assay sensitivity.

**Tone-dependent fear conditioning**

To evaluate the specificity of the fear-conditioning impairment observed, we tested mutant and wild-type mice in the tone-dependent fear conditioning task, which does not require hippocampal function in rodents. In this task, a 20-second long tone was presented immediately prior to each footshock during the conditioning phase of the experiment (Figure 5C). In the testing phase of the experiment, animals were placed in a novel cage and subsequently the tone was presented (Figure 5D). Prior to the tone, mutant (n=8) and wild-type (n=8) animals did not freeze significantly in the novel cage. When the tone was

presented, both wild-type and mutant animals displayed freezing behavior, and the two groups did not differ significantly (p=.914).

## DISCUSSION

We have shown that PKCγ mutant mice can learn to carry out two learning tasks, the hidden-platform Morris water maze and context-dependent fear conditioning, although mild to moderate deficits are evident. The hidden-platform Morris water maze and context-dependent fear conditioning tasks both have been shown to require the integrity of the hippocampus in rats (Morris, 1981; Kim et al., 1992). In light of the known similarity of hippocampal functions across species (Eichenbaum et al., 1992), it is likely that the hippocampus is also needed for performance by mice in the hidden-platform Morris water maze and context-dependent fear conditioning tasks. Both of these tasks involve the configuration or integration of multiple cues or facts, a process broadly termed configural or declarative learning. However, these two tasks differ considerably otherwise, both in terms of the motivation involved and in the elicited response. Thus, it is striking that similar results were observed in both tasks. These findings suggest that PKCγ mutant mice possess a mild deficit in configural or declarative learning per se rather than task-specific performance deficits.

Several additional results support the conclusion that the mild to moderate impairments we observed in these two tasks are due to learning impairments rather than task-specific performance deficits. Mutant mice reach the wild-type level of performance with training in the non-spatial visible-platform Morris water maze task. It is unlikely, therefore, that PKCγ mutant mice harbor a significant performance deficit such as a swimming impairment, lack of motivation to escape

the water, or poor vision. The deficit we observe in the hidden-platform Morris water maze task with PKCγ mutant mice is dependent on the training regimen, as we found that with more intensive training this deficit could be overcome (data not shown). Therefore, PKCγ mutant mice are capable of performing well in this task. In the context-dependent fear conditioning task, mutant mice exhibited normal freezing immediately following a shock (Figure 5A), indicating that the deficiency in the conditioned freezing response (Figure 5B) observed one day later is not likely to be a consequence of performance deficits associated with motor, sensory, or motivational factors. Furthermore, PKCγ mutant mice displayed normal freezing in the tone-dependent fear conditioning task, again arguing against a performance deficit. Finally, the context-dependent fear conditioning appears to be specific for the conditioned context, as freezing in an unconditioned, novel context was minimal (See Figure 6D; the first three minutes correspond to freezing in the novel context).

The accompanying paper (Abeliovich et al., 1993) shows that hippocampal LTP is abnormal in PKCγ mutants in that it is absent or greatly attenuated when induced in vitro by conventional tetanic stimulation, although apparently normal LTP can be enabled by prior low-frequency stimulation. Thus, our overall data show that LTP, as assessed by conventional tetanic stimulation, is not essential for mice to exhibit hippocampus-dependent learning capabilities (Table 1). However, the modified properties of hippocampal LTP correlate with mild to moderate deficits in spatial and contextual learning, consistent with the notion that LTP is a synaptic mechanism for these forms of learning. The learning deficits observed in PKCγ mutant mice may be causally related to the LTP modification.

Another interesting candidate synaptic mechanism for learning that has emerged from this and other recent studies is LTD. While there has been no direct evidence, LTD satisfies the same criteria for a synaptic learning mechanism as does LTP (Siegelbaum and Kandel, 1991). Furthermore, LTD appears to correlate with spatial and contextual learning capabilities, as it is intact in PKCγ mutant mice (Abeliovich et al., 1993), which display spatial and contextual learning, whereas it is impaired in αCaMKII mutant mice (Table 1, C. F. Stevens, S.T., and Y. Wang, unpublished observations), which are deficient in spatial learning (Silva et al., 1992b). It is also of note that AP5, which has been shown to impair spatial and contextual learning (Morris et al., 1986; Kim et al., 1992), is now known to block not only LTP but also LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992). It is therefore possible that the learning impairments observed in AP5 treated animals result from the disruption of both LTP and LTD.

Although protein kinase C has been implicated in synaptic plasticity and in learning and memory, previous studies rarely addressed the functional significance of specific PKC isotypes. It had been hypothesized that PKCγ might serve a specific function in the CNS because of its unique expression pattern among the PKC isotypes (Nishizuka, 1988). Van der Zee *et al.* (1992) found that the level of PKCγ immunoreactivity in the CNS correlates with spatial learning, consistent with a role for PKCγ in spatial learning. Our data indicate that PKCγ does play a role in both LTP and in learning and memory, but is not essential for either process. One possible interpretation of these findings is that other PKC isotypes, such as the more abundant PKCβ isotypes, can complement for the PKCγ deficiency. However, this is clearly not always the case, as conventionally induced LTP is severely deficient in the PKCγ mutant mice. Therefore, we find it

likely that PKCγ possesses certain unique functions. Another and perhaps more interesting interpretation of these findings is that second messenger systems (Abeliovich et al., 1993; Silva et al., 1992a; Silva et al., 1992b) play a regulatory role in LTP and in contextual learning, so that the deficits we have observed reflect a failure in the regulation of LTP and contextual learning rather than a deficit in their mechanisms. Our data suggest a role for the γ isotype of PKC in the regulation of contextual learning.

It is now of interest to further characterize the physiological differences between PKCγ and αCaMKII mutant mice in order to identify additional candidate mechanisms and anatomical regions that may be involved in the different behavioral phenotypes observed in these mutants. It is also of interest to determine whether these different mutations function within the same genetic pathway by generating mice that harbor both mutations.

## MATERIALS AND METHODS

### Animals

Animal care was in accordance with institutional guidlines. Animals were typed for the PKCγ genotype by tail biopsy (Abeliovich et al., 1993). Animals utilized were of the 129/Ola X C57BL/6 or 129/Ola X Balb/C genetic backgrounds, as stated. Albino animals of the 129/Ola X C57BL/6 were not utilized to avoid the possiblity of visual impairments. Mice were housed in standard animals cages in an animal facility with a 12 hour light/dark cycle: lights on at 0700. Standard laboratory chow and water was provided ad lib.

### Morris water task

Naive adult mice (6-10 weeks old) were utilized. The training apparatus was a circular, polypropylene (Nalgene) or galvanized steel pool 120 cm in diameter. The water was maintained at 26$^{o}$C. Non-toxic Crayola paint was added to make the water opaque and to blend with the color of the pool wall. The pool was located in a laboratory room that had a number of items that could be seen by an animal swimming in the pool (for details see Paylor et al., 1993).

### Visible-platform training

The visible platform was 11.5 X 11.5 cm in diameter. A cylinder (13 cm tall and 6.5 cm in diameter) was attached to the top of the platform such that the bottom of the cylinder was 10 cm above the platform top. The top of the platform was 1-cm below the surface of the water. Prior to training, each mouse was acclimated to

the water and escape platform by placing it on the platform for 15 seconds followed by a 15 second swim and 3 practice climbs onto the platform. The platform location varied among four possible places within each block of four trials. A trial was started by placing an animal along the edge of the pool facing the wall in one of four start locations. A subject was allowed 60 seconds to locate the platform. Animals not finding the platform in 60 seconds were guided there by the experimenter. Animals were allowed to remain on the platform for 20 seconds. Each mouse was given 12 trials a day, in blocks of four trials for three consecutive days (massed-trial procedure). The time taken to locate the escape platform (escape latency) was determined on each trial. Mice in these visible-platform experiments started training either on the visible-platform task first followed by training on the hidden-platform task, or they were started on the hidden-platform task followed by visible-platform training. Only the latter data are presented in the results.

**Hidden-platform training**

Data presented in this study from the hidden-platform experiments were obtained using the same procedures as that used for the visible-platform task with the following exceptions. First, there was no visible cylinder attached to the platform. Second, the platform location varied among different animals, but always remained in the same place for any given subject. Finally, mice were given 4 trials a day for 9 consecutive days. Each trial on a day was separated by 1 hour (distributed-trial procedure). During training escape latencies were determined.

One-hour after the last training trial, each animal was given a probe trial. During the probe test, the platform was removed and each animal was allowed 60 sec to search the pool. Two measures of search behavior were determined. A quadrant search time measure was obtained by dividing the pool into four equal

quadrants, and determining the amount of time spent in each quadrant . A platform crossing measure was obtained by counting the number of times a subject crossed the exact place in the training quadrant where the platform had been located during training. For comparison, the number of times a subject crossed the equivalent location in each of the other quadrants was determined.

Following the probe trial mice were given a random-platform test. On day 1 of the random-platform test, animals were given 4 trials with the platform in its original location. On the next day subjects were given 1 trial with the platform in its original location and three trials with the platform in one of the platform sites in the other three quadrants. The average time taken to locate the platform on the trials prior to the platform being relocated in a new location was used as an animals "original" platform escape latency and the average time taken to locate the platform when it was in its 3 new locations was used as an animals "new" platform escape latency.

**Fear conditioning**

Naive adult mice (6-10 weeks old) were housed individually for at least 1 week prior to behavioral testing. Mice were handled routinely for one week prior to behavioral experiments to reduce stress. Fear conditioning and testing was conducted in a small rodent chamber (Coulbourn) containing a stainless steel rod floor (5 mm diameter, spaced 1 cm apart) through which scrambled footshocks could be administered. The chamber was placed inside a sound-attenuating chest (Coulbourn) with a ventilation fan providing background noise. The chamber was cleaned with 1 % acetic acid and dried completely before each animal was placed inside. Freezing was assessed by a time-sampling procedure in which an observer blind to mouse genotype scored each mouse every 2

seconds. Percent freezing was calculated per minute. Experiments were recorded on videotape.

**Context-dependent fear conditioning**

In the conditioning phase, animals were placed in the shocking chamber for 3 minutes and subsequently subjected to three footshocks (0.5 mA intensity, 1 second duration, 1 minute apart). Mice were removed from the chamber one minute after the last footshock. In the testing phase (the next day), animals were returned to the shocking chamber and freezing was monitored for 8 minutes. Preliminary experiments indicated that these conditions (the interval between the conditioning phase and the testing phase, the chamber, and the shock intensity) are sufficient for observing a freezing impairment (data not shown).

**Tone-dependent fear conditioning**

Animals were placed in the shocking chamber for three minutes and then presented with 3 x 20 second loud tones (approximatey 75 db, 1000 Hz, 3 minutes apart) through a speaker mounted on the chamber. A footshock (0.5 mA intensity, 1 second duration) was presented at the offset of each tone. Mice were removed from the chamber one minute after the last footshock. In the testing phase (the next day), mice were placed in an empty plastic cage different fron the shocking chamber (to minimize freezing due to context-dependent fear conditioning) and freezing was scored for 3 minutes prior to and subsequently for 8 minutes in the presence of the tone.

**Data Analysis**

Both male and female mice were tested, but since there were no significant gender differences these data were grouped together. The data were analyzed

by analysis of variance (ANOVA). Simple-effects analysis and Newman-Keuls analysis were used for post-hoc comparisons.

## ACKNOWLEDGEMENTS

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# FIGURES

**Figure 1.** Average time taken to locate the visible platform (escape latency) for wild-type and PKCγ mutant mice. Mice were trained using a massed-trial (12 trials/day) procedure. These data are from mice previously trained on the hidden-platform task. Performance improved for each genotype [$F(8,104) = 15.547$, $p < .0001$], and there was no significant difference between wild-type and mutant mice [$F(1,13) = 2.737$, $p = .122$].

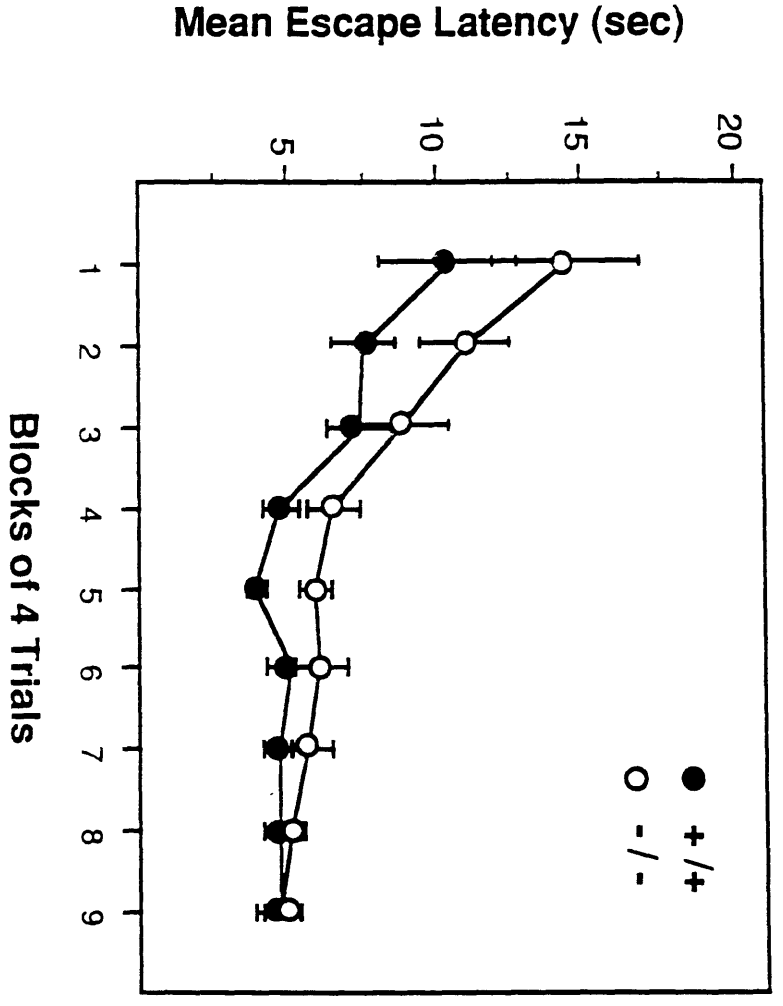
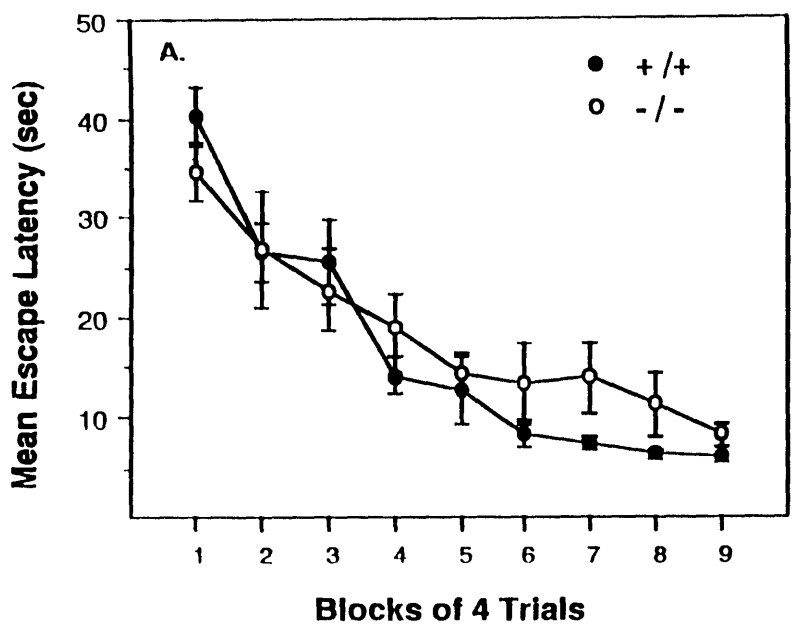

Mean Escape Latency (sec)
5
10
15
20
Blocks of 4 Trials
1
2
3
4
5
6
7
8
9
+ /+
- /-

**Figure 2.** Performance of wild-type and PKCγ mutant mice trained on the hidden-platform version of the Morris water task. Mice were trained using a distributed-trial (4 trials/day) procedure. (A) Average escape latency during training. Performance of wild-type and mutant mice improved during training [$F(8,152) = 34.695$, $p < .0001$], and there was no difference between the two genotypes [$F(1,19) = 0.286$, $p = .5987$]. (B) Average time subjects spent in each quadrant of the pool during the probe test. (C) Mean platform crossings during the probe trial. Wild-type mice spent more time in the training quadrant compared to the other quadrants [$F(3,24) = 44.777$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .01$] and crossed the site where the platform was located more often than the alternate sites [$F(3,24) = 57.517$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .01$]. Similarly, mutant mice spent more time in the training quadrant than the other quadrants [$F(3,33) = 20.988$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .01$] and crossed the site where the platform was located more often than the alternate sites [$F(3,33) = 13.251$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .01$]. Wild-type mice did not spend any more time in the training quadrant compared to mutant mice [$t(19) = 1.145$, $p > 0.26$], but did cross the correct site more often than the mutants [$t(19) = 2.337$, $p < 0.031$]. (D) Performance on the random platform test. Both wild-type and mutant mice located the platform more rapidly when it was in its original training site compared to when it was located in a new site [$F(1,19) = 114.045$, $p < .0001$], and there was no difference between the two genotypes [$F(1,19) = 0.001$, $p = 0.9846$].


A.
+/+
-/-
Mean Escape Latency (sec)
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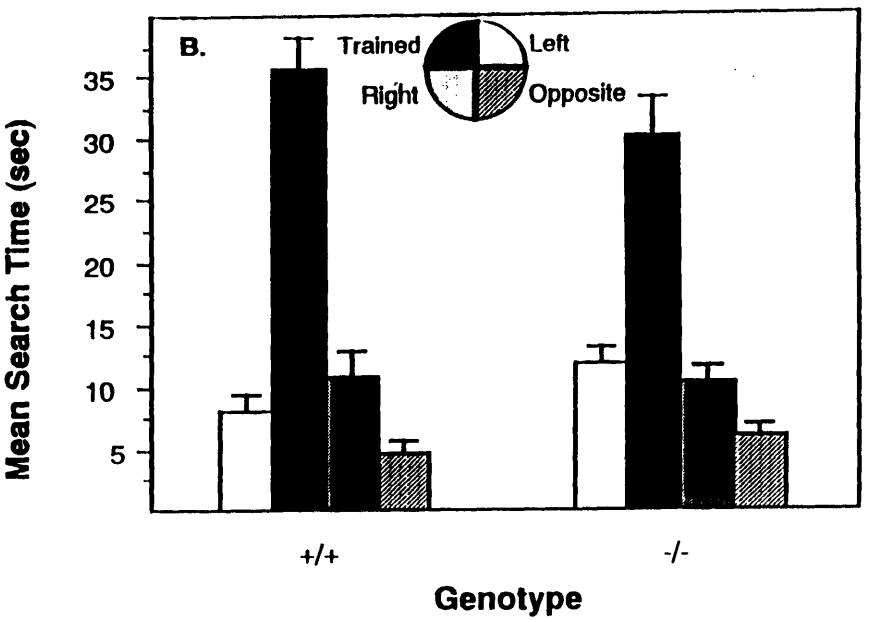
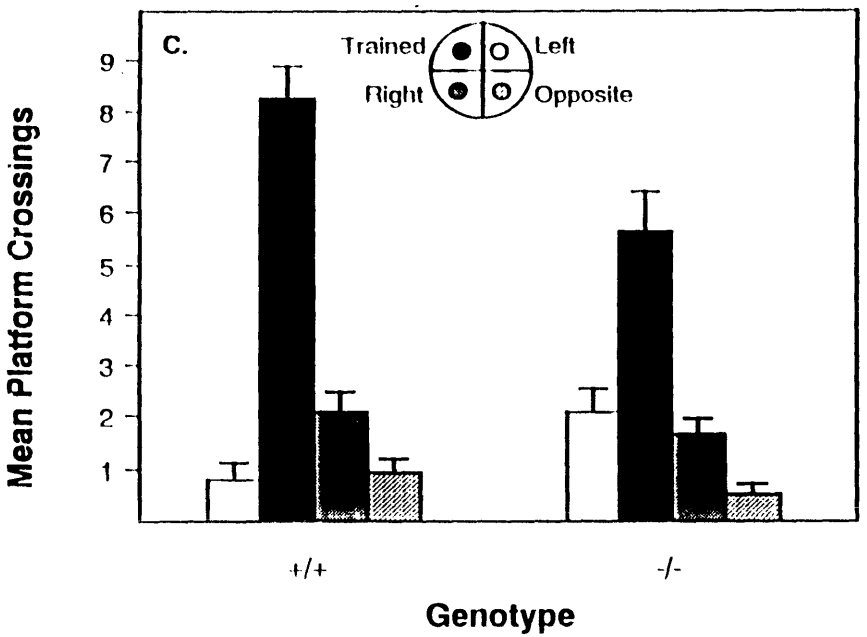
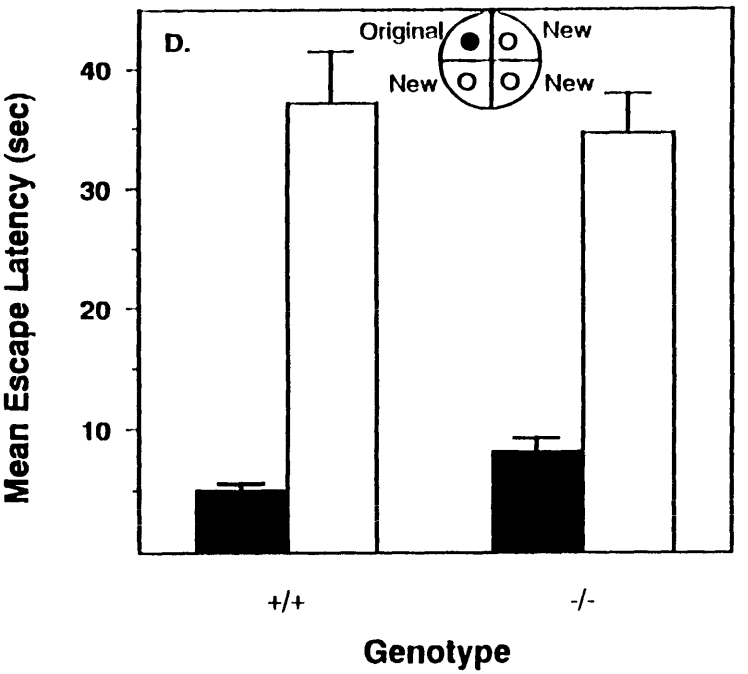
40
30
20
10
1
2
3
4
5
6
7
8
9
Blocks of 4 Trials


B.
Trained
Left
Right
Opposite
Mean Search Time (sec)
35
30
25
20
15
10
5
+/+
-/-
Genotype


C.
Trained
Left
Right
Opposite
Mean Platform Crossings
9
8
7
6
5
4
3
2
1
+/+
-/-
Genotype


D.
Original
New
New
New
Mean Escape Latency (sec)
40
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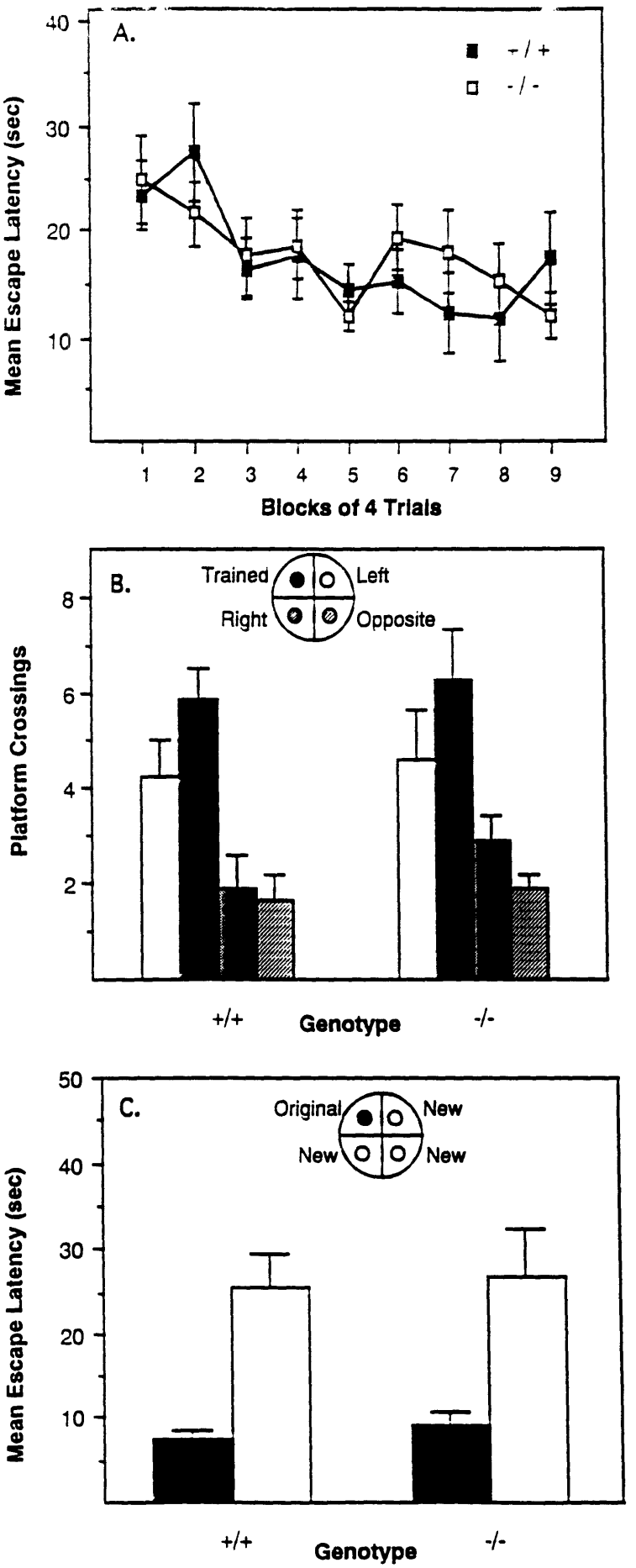
10
+/+
-/-
Genotype

**Figure 3.** Performance of wild-type and PKCγ mutant mice trained on the hidden-platform version of the Morris water task. Mice of the 129/Ola X Balb/c genetic background were trained using a massed-trial (12 trials/day) procedure. These data are from animals previously trained on the visible-platform task (A) Average escape latency during training. Performance of wild-type and PKCγ mutant mice improved during training [$F(8,104) = 3.90$, $p = .0005$], and there was no difference between the two genotypes [$F(1,13) = 0.012$, $p = .9132$]. (B) Mean platform crossings during the probe trial. Wild-type mice crossed the site where the platform was located more often than the alternate sites [$F(3,21) = 15.63$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > Left > Right and Opposite quadrants, $p < .05$]. Similarly, mutant mice crossed the site where the platform was located more often than the sites in the opposite and right quadrants [$F(3,28) = 5.099$, $p = .0099$. Newman-Keuls post-hoc comparison: Trained > Right and Opposite quadrants, $p < .05$]. Wild-type mice did not cross the correct site more often than the mutants [$t(13) = -0.341$, $p < .65$]. (C) Performance on the random platform test. Both wild-type and PKCγ mutant mice located the platform more rapidly when it was in its original training site compared to when it was located in a new site [$F(1,13) = 24.167$, $p = .0003$], and there was no difference between the two genotypes [$F(1,13) = 0.165$, $p = .6914$].


A.
-/+
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Mean Escape Latency (sec)
40
30
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1
2
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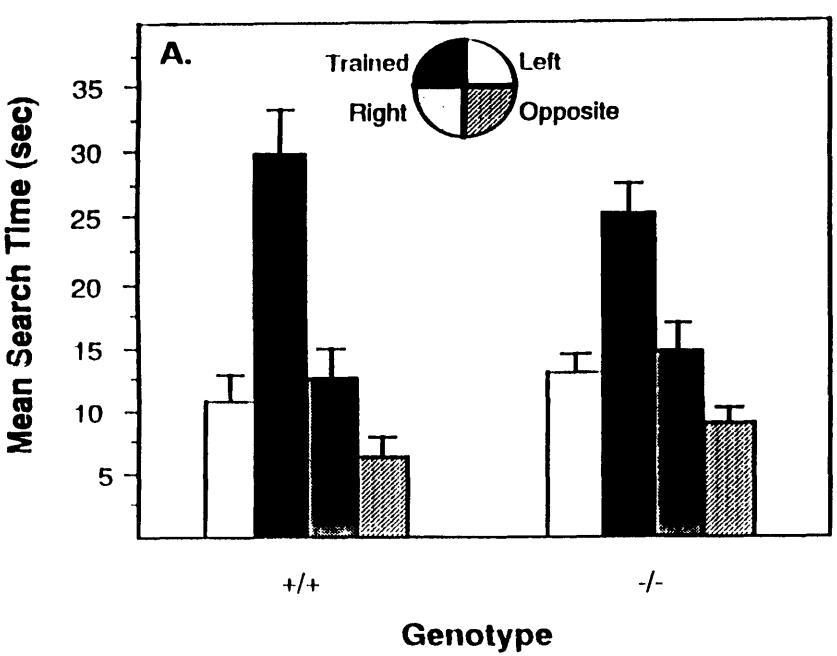
4
5
6
7
8
9
Blocks of 4 Trials
B.
Trained
Left
Right
Opposite
Platform Crossings
8
6
4
2
+/+
Genotype
-/-
C.
Original
New
New
New
Mean Escape Latency (sec)
50
40
30
20
10
+/+
Genotype
-/-

**Figure 4.** Performance of wild-type and PKCγ mutant mice on the 4-week retention probe test. These data are from the mice trained with the distributed-trial procedure. (A) Average time subjects spent in each quadrant of the pool during the retention probe test. (B) Mean platform crossings during the retention probe trial. Wild-type mice spent more time in the training quadrant compared to the other quadrants [$F(3,24) = 13.78$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .01$] and crossed the site where the platform was located more often than the alternate sites [$F(3,24) = 17.629$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .01$]. Similarly, mutant mice spent more time in the training quadrant than the other quadrants [$F(3,33) = 7.743$, $p = .0005$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .01$] and crossed the site where the platform was located more often than the alternate sites [$F(3,33) = 7.033$, $p < .0001$. Newman-Keuls post-hoc comparison: Trained > All other quadrants, $p < .05$]. Wild-type mice did not spend any more time in the training quadrant compared to mutant mice [$t(19) = 1.3.18$, $p < 0.2$], but did cross the correct site more often than the mutants [$t(19) = 2.159$, $p < .044$].


A.
Trained
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Mean Search Time (sec)
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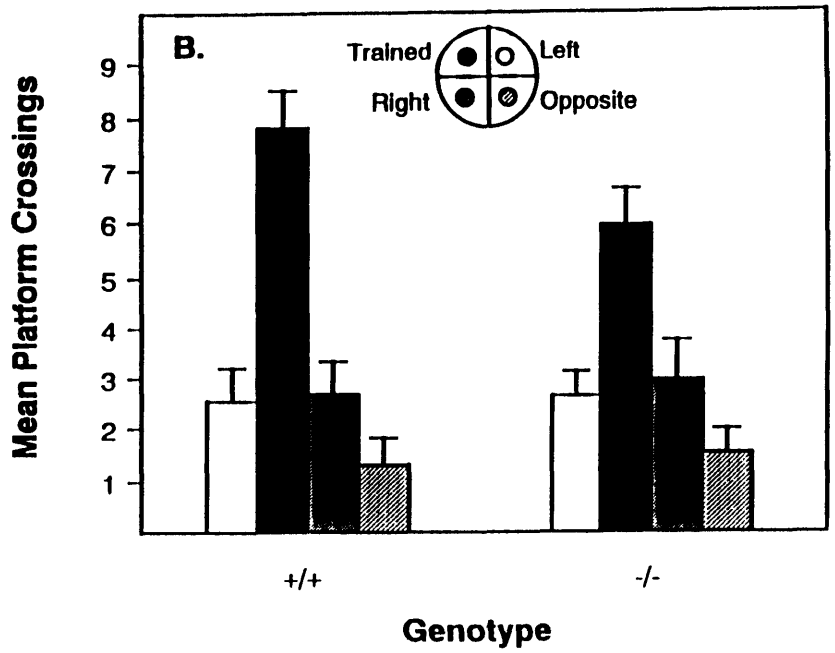
B.
Trained
Left
Right
Opposite
Mean Platform Crossings
9
8
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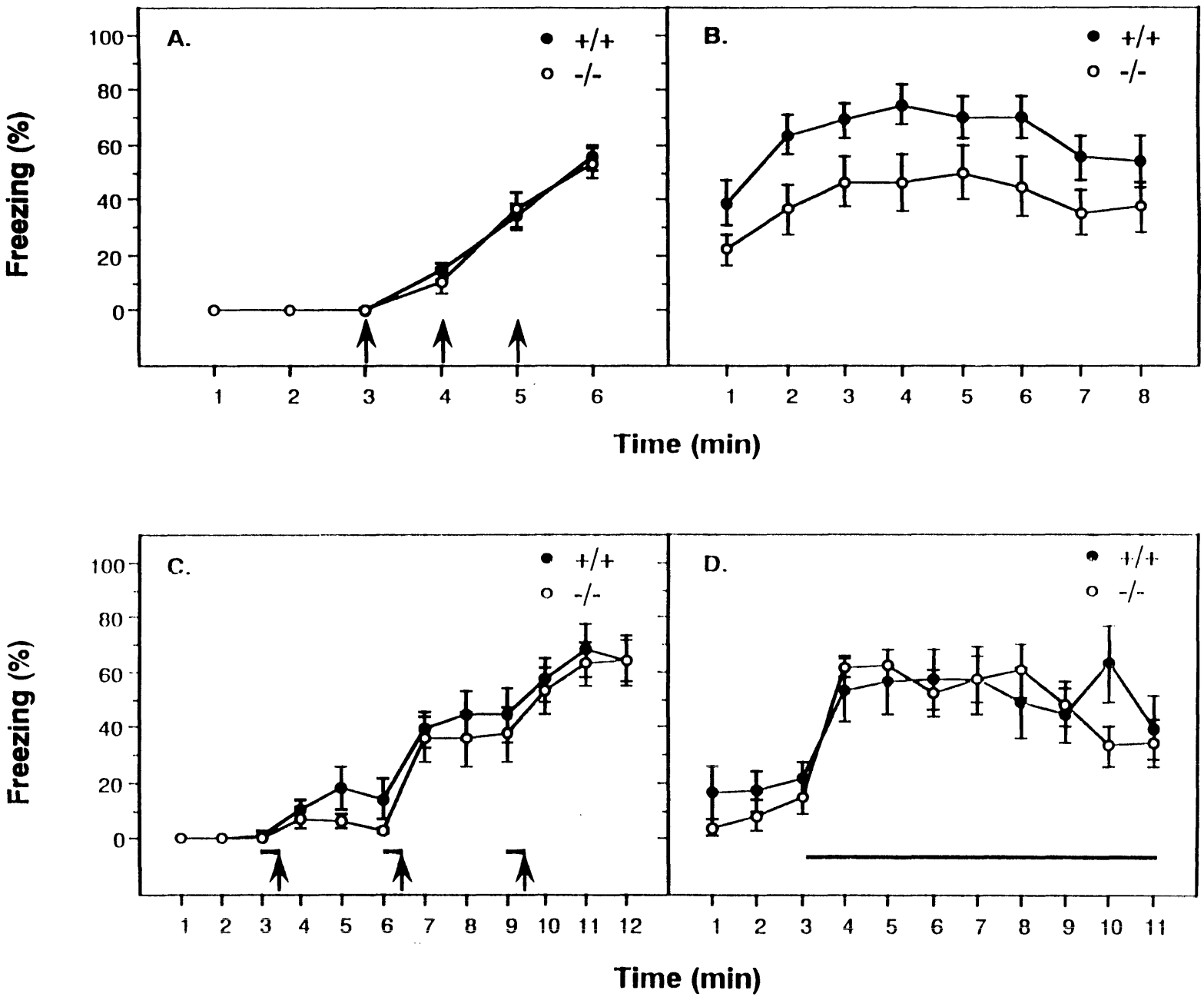
2
1
+/+
-/-
Genotype

**Figure 5**. Mean percent freezing in the context-dependent and tone-dependent conditioning tasks. (A) Context-dependent fear conditioning, conditioning phase. Wild-type and PKCγ mutant mice displayed a comparable degree of freezing [$F(1,25)= 4.759$, $p=.864$] immediately after the footshocks (arrows). (B) Context-dependent fear conditioning, testing phase. Mutant mice displayed significantly less freezing than wild-type mice when returned to the shocking chamber the next day [$F(1,25) = 4.525$, $p=.043$]. (C) Tone-dependent fear conditioning, conditioning phase. Wild-type and PKCγ mutant animals displayed a comparable degree of freezing [$F(1,14)= .460$, $p=.5987$] immediately after the footshocks (arrows) at the offset of the tone (solid line). (D) Tone-dependent fear conditioning, testing phase. Wild-type and mutant mice displayed a comparable degree of freezing [$F(1,14) = 0.012$, $p=0.9142$] when presented with the tone (solid line) in a novel context the next day.


A.
B.
C.
D.
+/+
-/-
Freezing (%)
Time (min)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12

# TABLE

**Table 1.** Summary of data from mutant and AP5 treated mice. *, partial impairment; ND, not done. LTP and LTD in PKCγ mutant mice is from Abeliovich et al. (1993); LTP in αCaMKII mutant mice is from Silva et al. (1992a); LTD in αCaMKII mice is from C. Stevens, S.T., and Y. Wang, unpublished observations; Learning in αCaMKII mice is from Silva et al. (1992b); AP5 LTP is from Collingridge and Singer (1990); AP5 LTD is from Dudek and Bear (1992); AP5 learning is from Morris et al. (1986).

| | PKCγ -/- | αCaMKII -/- | AP5-treated |
|---|---|---|---|
| LTP- conventionally induced | - | - | - |
| LTP- primed | + | - | ND |
| LTD | + | - | - |
| Learning-spatial/ contextual | +* | - | - |

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# CHAPTER 4

# On the Genetic Analysis of Mammalian Learning and Memory

The genetic basis of mammalian learning and memory remains to be elucidated. A conventional genetic screen for behavioral mutants has not proven to be practical in mammalian systems. An alternative approach involves generating targeted mutations in genes implicated in learning and memory by previous physiological or biochemical studies. The gene knockout technique has been applied to genes implicated in learning and memory, and the behavioral phenotypes of three targeted mutations -- αCaMKII, fyn, and PKCγ -- have been analyzed to date (Silva et al., 1992a, 1992b; Grant et al., 1992; Abeliovich et al., 1993a, 1993b). αCamKII, fyn, and PKCγ all appear to be involved in hippocampal LTP, a putative physiological substrate for learning and memory, and the three corresponding mutant mouse strains all display deficits in spatial learning, a form of learning that is dependent on the integrity of the hippocampus formation. These experiments have served to correlate synaptic plasticity in the hippocampus with a hippocampus-dependent learning process, spatial learning. Furthermore, they have demonstrated the utility of the gene knockout technique in studies of mammalian learning and memory. However, it is fair to question whether they have significantly advanced our understanding of learning and memory. The analysis of behavioral phenotypes in genetically altered mice is complex, as a learning deficit may be confounded by indirect or pleotropic effects, such as developmental abnormalities. This chapter describes limitations to and future prospects of the generation and behavioral analysis of mouse mutant strains.

**Specificity of Targeted Mutations.** Targeted mutations can serve as extremely specific pharmacological agents that precisely disrupt an enzymatic activity. However, the gene knockout technique is imprecise relative to pharmacological manipulations in that a given genetic lesion may be expressed anywhere in the animal, and at any time during the development of the animal. Thus, it may be difficult to define the exact nature of the deficit in a knockout mouse, or to ascribe the deficit directly to the loss of a physiological process that underlies learning and memory, rather than to an indirect effect. For example, fyn mutant mice (Grant et al., 1992) display a spatial learning deficit as well as developmental abnormalities in the hippocampus. Thus, it is not obvious whether the learning deficit displayed by fyn mutant mice reflects a role for fyn in learning and memory processes per se, or results from the developmental deficit observed. Although no such developmental abnormalities have been observed at the light microscopic level in the hippocampus of PKCγ or αCamKII mutant mice, it is nonetheless possible that subtle developmental abnormalities also underlie the behavioral phenotypes observed in these other mutant strains.

Fyn, αCamKII, and PKCγ are all expressed in several CNS regions implicated in spatial learning processes, including the hippocampus, the frontal cortex and the entorhinal area. Because of the widespread expression of these genes, a correlation between an observed physiological abnormality present in a specific CNS region, such as deficient LTP in the CA1 region of the hippocampus, and a learning deficit, such as deficient spatial learning, may be misleading; there may be additional physiological abnormalities in the hippocampus or elsewhere that are responsible for the learning deficit. Thus, it may be necessary to analyze physiological properties throughout the

hippocampus and elsewhere in the CNS in order to correctly identify important physiological mechanisms for spatial learning.

The fyn, αCamKII, and PKCγ mutations may also lead to non-learning deficits that confound behavioral tests. For example, fyn, αCamKII and PKCγ are all expressed in areas important for visual processing, and a visual impairment may therefore confound the behavioral analyses of these mice. Furthermore, PKCγ is expressed in the cerebellar cortex, a structure involved in motor coordination, and therefore physical disabilities may confound a behavioral analysis. Finally, fyn is also expressed outside of the CNS, and therefore may lead to general malaise (although this is not evident), which may confound a behavioral test. Thus, it is important to exclude non-learning impairments as underlying an apparent behavioral deficit.

**Behavioral Analysis of Gene Targeted Mice**. The issue of specificity is central to the analysis of learning impairments, be they due to lesions, pharmacological agents, or genetic manipulations (Weiskrantz, 1968). To analyze the specificity of a learning deficit, it is most common to evaluate performance in two or more tasks that are operationally very similar in terms of physical or non-cognitive skills, but that differ with respect to the type of learning they assay. In fact, such assays often serve to define a form of learning. An example of this is found in the Morris water maze hidden- and visible- platform tasks, which are used to evaluate spatial learning in rodents (Morris, 1981). In the hidden platform version of the Morris water maze task, animals are placed in an opaque pool of water at one of 4 start sites, and a submerged platform is kept at another site in the pool throughout training. In order to escape the water, rodents must learn to navigate to the hidden platform by mapping its position relative to visual cues outside of

the pool. In the visible-platform Morris water maze task, a cylindrical landmark is placed on the escape platform, indicating its position. Animals are placed in the pool at one of four start sites but, unlike the hidden-platform, the visible platform is relocated to new quadrants of the pool between trials. Therefore, the rodents must learn to associate the landmark with the location of the platform, and spatial information is irrelevant. The two tasks are very similar in terms of motivation and physical requirements, such as swimming and climbing, and thus if an impairment is observed in the hidden platform but not the visible platform task, it is likely due to a specific spatial learning deficit.

However, the specificity of an impairment cannot definitively be concluded by the Morris water maze tasks, as there exist subtle non-cognitive differences between the visible-platform and the hidden-platform tasks that may also differentiate performance in these two tasks. For instance, performance of mice on the visible platform task is measured in terms of the time taken to reach the platform over the course of training (latency), whereas performance on the hidden-platform task is for the most part evaluated in a probe test, in which the platform is removed and the search strategy of the mice is measured over a minute. The specificity of a behavioral deficit would be more correctly evaluated if the two tasks employed a similar assay. This could be achieved by performing a probe test for the visible platform task as well, in which the platform would be removed, the flag placed in a quadrant of the pool, and the search strategy of the mice evaluated over the course of a minute.

There exist additional possible confounding factors in comparing performance in the visible-and the hidden- platform tasks. For instance, a visual impairment such as nearsightedness would be more likely to effect the hidden

platform task, which requires viewing objects outside of the pool, than the visible platform task, which requires viewing a flag placed on the platform. This may be addressed either by bringing the multiple "distant" objects more closely in the hidden-platform task, or by placing the visible flag further from the platform.

The hidden-platform and the visible-platform Morris water maze tasks also differ in terms of the amount of training required to perform well in these two tasks. Therefore, a mutant mouse may perform well in one task and poorly in the other relative to wild-type mice because of a difference in the degrees of difficulty of these two tasks. This could be addressed by employing multiple training schemes with varying degrees of difficulty for each task.

Confirmation of a hippocampus-dependent learning impairment can be obtained by demonstrating the impairment in multiple hippocampus-specific tasks. If these tasks differ significantly in terms of their motivational and physical requirements, then many task-specific impairments can be excluded as possible confounding factors. For example, PKCγ mutant mice display a mild deficit in two hippocampus-specific tasks, the Morris water maze hidden-platform task and context-dependent fear conditioning. In context-dependent fear conditioning, rodents learn to associate a footshock with a distinctive cage environment, and consequently freeze when presented with the same distinctive cage environment on subsequent trials, even in the absence of the footshock (Kim et al., 1992). As the hidden-platform Morris water maze and context-dependent fear conditioning tasks differ significantly in terms of their motivational and physical requirements (but are both hippocampus-dependent), it is likely that the performance of PKCγ mutant mice in these tasks is due to a hippocampus-dependent learning deficit rather than a task-specific deficit.

**Future Prospects for the Genetic Analysis of Mammalian Learning and Memory**. Given the phenotypes observed in the αCamKII, fyn and PKCγ mutant mice, the prospect of additional mutant mice analyses is quite exciting. However, it seems worthwhile to suggest some basic guidelines for the behavioral analysis of mutant mice, especially with respect to hippocampus-dependent learning, so as to accommodate the comparison of data from future studies.

The genetic background of a mutation can clearly affect the analysis of behavioral phenotype (Wehner et al., 1990), and it is therefore important to standardize the genetic background used. It appears likely that the sensitivity of a behavioral assay would best be served by "smart" genetic backgrounds that perform well in hippocampus-dependent learning tasks, such as C57BL/6 or non-inbred backgrounds. It is generally useful to employ inbred strains of mice in genetic analyses in order to minimize genetic variability, but there is no evidence that homozygote animals perform less variably than heterozygote animals or their progeny (Wehner et al., 1990), suggesting that many genes are involved in the strain background differences observed (Wright, 1968). A technical limitation exists because most knockout mutations are generated in the 129/Ola or 129/Sv genetic backgrounds, and these genetic backgrounds probably perform relatively poorly in the Morris water maze hidden-platform task (Grant et al., 1992.; 129/Ola mice have unpigmented eyes and may display visual impairments). It therefore seems most efficient to employ 129/Ola x C57BL/6 (generated by initially crossing mutants of the 129/Ola background with C57BL/6 mice and intercrossing the progeny, as in the analysis of PKCγ mutant mice) or 129/Sv x C57BL/6 non-inbred genetic backgrounds for the initial behavioral analyses of mutant mice. Subsequently, inbred mutant mice in the C57BL/6 genetic

background, generated by repeatedly backcrossing the mutation into the C57BL/6 genetic background (or by creating the mutation in the C57BL/6 background using C57BL/6-derived stem cells) should be analyzed as well. The assays used in the behavioral analyses of mutant mice should also be standardized in future studies. If the 129/Ola x C57BL/6 genetic background is employed, the assays described for the behavioral analysis of PKCγ mutant mice may be employed.

Some of the limitations described above for conventional targeted mutant mice may be overcome in the future with the use of more precise genetic disruptions that specify the timing or location of the genetic lesion generated. Regulated gene knockout strategies have been described (Gu et al., 1993). The regulated expression of a foreign, site-specific recombinase (an enzyme that cuts and pastes DNA) can generate specific deletions in genes that have been appropriately tagged with sites that the recombinase recognizes. By specifying the timing and location of the expression of the recombinase, the nature of the genetic lesion can be controlled. Thus, concerns such as possible developmental or non-hippocampal deficits in the behavioral analysis of αCamKII, fyn, and PKCγ mutant mice could be eliminated

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# APPENDIX I

# On Somatic Recombination in the Central Nervous System of Transgenic Mice

**Asa Abeliovich, David Gerber, Osamu Tanaka, Motoya Katsuki, Ann M. Graybiel, and Susumu Tonegawa***

A. Abeliovich and D. Gerber, Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, 02139.

O. Tanaka, Department of Anatomy, Shimane Medical University, Izumo, Japan.

M. Katsuki, Central Institute for Experimental Animals, Kawasaki, and Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan.

A. M. Graybiel, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139.

S. Tonegawa, Howard Hughes Medical Institute at Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

# APPENDIX II

# Cyclophilins in *C. elegans*

Michael O. Hengartner[#]
Asa Abeliovich*[#]
Susumu Tonegawa*
H. Robert Horvitz

Address: Howard Hughes Medical Institute
Department of Biology, Room 56-629
Massachusetts Institute of Technology
77 Massachusetts Avenue
Cambridge, Massachusetts 02139, USA
Telephone: (617) 253-4671
FAX: (617) 253-8126

* Howard Hughes Medical Institute
Center for Cancer Research
Department of Biology
Room E17-353
Massachusetts Institute of Technology
77 Massachusetts Avenue
Cambridge, Massachusetts 02139, USA

# The first two authors contributed equally to this work.

**Summary**

We have cloned and characterized cyclophilin genes in *C. elegans.* Five cyclophilin genes have been identified so far, more than in any other species. The physical location of several of these have been determined. We have also isolated and mapped a *C. elegans* calcineurin A gene.

**Introduction**

The immunosuppressant Cyclosporin A (CsA) is the most widely used drug to prevent graft rejection in organ transplants, and it has been credited with initiating a revolution in the type of transplantations that can be performed and in the frequency and success rate of transplantations in general (Kahan, 1989). CsA-mediated immunosuppression is mainly the result of an inhibition of T cell activation (reviewed by Walsh et al., 1992). Cyclosporin A binds with high affinity to a cellular protein called cyclophilin (CyP), which is identical peptidyl-prolyl *cis-trans* isomerase, an enzyme that catalyzed isomerization between the two proline conformations in peptides (Fisher et al., 1989; Takahashi et al., 1989).

Cyclophilins are highly conserved among eukaryotes, from yeast to man (Trandinh et al., 1992). Several cyclophilins exist in a given species, and these differ in their ability to bind CsA and in their subcellular localization. For example, one form is found in the cytosol (CypA), a second in the secretory pathway (CypB), and a third in mitochondria. Due to the nature of their enzymatic activity, it has been suggested that the normal function of CyPs might be to act as chaperonins, facilitating the folding of nascent polypeptides by accelerating the isomerization of prolyl residues into the right isomer (Heitman et al., 1992).

CsA behaves as a CyP substrate analog and binds to the active site of the protein, resulting in a competitive inhibition of the CyP isomerase activity. However, the biological activity of CsA is not due to inhibition of the CyP isomerase activity, as CsA variants that are biologically inactive but still inhibit CyP can readily be synthesized (Shreiber, 1991), and inhibition of T cell activation occurs at CsA concentrations roughly 10 times lower than those required to inhibit CyP isomerase activity (Bierer et al., 1990). Rather, the ultimate target of CsA was found to be another enzyme, the protein phosphatase calcineurin (Friedman and Weissman, 1991; Liu et al., 1991). Binding of CsA to CyP causes a conformational change in the CsA molecule (Wüthrich et al., 1991); the resulting CsA-CyP complex (but not free CsA) is then able to bind and inhibit calcineurin activity .

Surprisingly, calcineurin inhibition by a CyP-CsA complex is a conserved mechanism of CsA action, as CsA-mediated toxicity in yeast is also mediated through CyP and calcineurin. The conservation between yeast and mammals of what appears *a priori* to be a rather strange and contorted mechanism of action suggests that CsA might subvert a normal signal transduction pathway that involves CyP and calcineurin, possibly by mimicking a cellular equivalent of CsA. In order to understand the various biological roles that cyclophilins play, we have initiated a study of these proteins in the genetically tractable metazoan *C. elegans*.

## Results

### Cloning of worm cyclophilins

We designed degenerate polymerase chain reaction (PCR) primers from regions conserved among vertebrate and yeast cyclophilins and used them to amplify nematode cyclophilin homologs. Two distinct bands could be amplified from *C. elegans* genomic DNA. We cloned the products corresponding to these bands and found that they encoded two distinct *C. elegans* cyclophilin genes, which we have named *cyp-1* and *cyp-2* (for *cyclophilin*). Three additional cyclophilin genes were identified by others in unrelated endeavors and generously provided to us: *cyp-3* was identified both by Mike Krause and Dick McCombie, *cyp-4* by Jim Waddle, and *cyp-5* by Dick McCombie (see below). Thus *C. elegans* contains at least five cyclophilin genes, which is more than are currently known for any other species. The physical location of the genes we have mapped so far are shown in Figure 1.

***cyp-1 V***

*cyp-1* was mapped to chromosome *V* by hybridization of the isolated PCR fragment to the *C. elegans* YAC polytene filter (The YAC polytene filter consists of an ordered set of YAC filter covering most of the *C. elegans* genome; Coulson et al., 1991). We localized *cyp-1* to a region covered by the YACs Y14B8 and Y49B3 (Figure 2a). There is a gap in the cosmid coverage in this region. *cyp-1* probably lies in this gap, as none of the cosmids in this region hybridized to the *cyp-1* probe (data not shown). The physical location of *cyp-1* places the gene between the cloned genes *unc-112* (M. Gilbert, personal communication) and *sdc-3* (Klein and Meyer, 1993) (Figure 1). A few known mutations, such as *unc-61(e228)*, also map to this 1 Mb interval. We have not determined whether any of these mutations might be in *cyp-1*.

We have used the *cyp-1* PCR fragment to isolated two independent clones from a *C. elegans* cDNA library. One of these (clone B30) contained 9 residues identical to the SL1 splice leader at its 5', suggesting that this cDNA is full length and that *cyp-1* is usually *trans*-spliced. This cDNA is predicted to encode a 192 amino-acid protein (Figure 2b), which shows a high degree of similarity to other cyclophilins. CYP-1 is most similar to the main cytosolic (CypA) class of cyclophilins (Table 1).

***cyp-2 III***

*cyp-2* was mapped to a region on chromosome *III* (Figure 3a) covered by the YACs Y20A12 and Y26C10. Hybridization of the *cyp-2* probe to cosmids from this region revealed that the cosmid ZK526, but not the flanking cosmids ZK781 and W02C8 contains the gene (Figure 3b, c). We have cloned three independent *cyp-2* cDNAs, none of which full length. A combination of cDNA and PCR sequences were used to deduce a partial *cyp-2* sequence. The available sequence is sufficient to encode a 136 amino-acid protein and is predicted to lack about 100-200 bp of coding sequence at its 5' end (Figure 3d). The partial CYP-2 sequence, like CYP-1, is most similar to the CypA subclass of cyclophilins (Table 1).

Several known genetic loci (*bli-5*, *egl-35*, *osm-7*, *unc-64*, *unc-71*, and *unc-81*) map very roughly to the region containing *cyp-2* (Figure 3a). We have not tried to determine whether any of these corresponds to *cyp-2*, as the metric (correlation between the physical map and the genetic map) in this region is unknown. Furthermore, while the placement of *cyp-2* under Y20A12/Y26C10 is solid, the linkage between these two YACs and the only reliable genetic marker on the *cyp-2* contig (*unc-25*/GAD) is rather dubious; thus, *cyp-2* could in principle

lay elsewhere in the genome. Testing of the locus with respect to deficiencies known to take out the region (e. g. *tDf1* and *eDf2*) should be able to resolve this issue.

***cyp-3***

*cyp-3* was independently identified by Krause, who obtained in an screen for an unrelated gene (M. Krause, personal communication), and by McCombie et al. (1992) as part of the *C. elegans* cDNA sequence project. We have not yet determined the physical location of this gene. The two cDNAs isolated by McCombie et al. (1992) encode most, and possibly all of the protein (Figure 4 and R. McCombie, personal communication): the predicted size of the protein (189 amino acids) is comparable to that of other cyclophilins, but no in-frame stop codon is found upstream of the first methionine, leaving open the possibility that the *cyp-3* cDNAs isolated are not full-length and that the true start of the *cyp-3* coding sequence is further upstream. The CYP-3 protein, like CYP-1 and CYP-2, is most similar to CypA cyclophilins (Table 1).

***cyp-4 II***

*cyp-4* was identified by Waddle during the characterization of the *cap-2 II* actin binding protein (J. Waddle and R. Waterston, personal communication). *cyp-4* and *cap-2* are neighbors: both genes are located on cosmid M106 on chromosome *II* (Figures 5a and 5b), and the open reading frames of the two genes are separated by only 618 bp (Figures 5c and 5d). *cap-2* has been precisely mapped through deficiency PCR mapping to a small interval on the genetic map that contains no known mutations (Figure 5a; T. Bogaert and J. Waddle, personal communication). Extensive genetic screens for lethal mutations in this interval have failed to identify any such mutations (J. Waddle,

personal communication). *cyp-4* is resides in the same interval as *cap-2* (Figure 5b), and thus there are no good candidate *cyp-4* alleles.

The protein encoded by the *cyp-4* locus (Figure 5e) is a rather divergent member of the cyclophilin family, showing for example only 39 % identity with human CypA (Table 1). CYP-4 is also unusual among cyclophilins in that it possesses a long hydrophobic extension at its C-terminus. A similar extension can be found in only two other cyclophilins: *Drosophila* ninaA and *S. cerevisiae* Cyp4p. Interestingly, the latter two proteins are also very divergent members of the family. However, the low degree of similarity between CYP-4, ninaA, and Cyp4p argues against them being direct homologs. A more likely hypothesis is that the C-terminal extension is indicative of a functional, rather than evolutionary, connection. The *ninaA* cyclophilin is required for proper folding in the endoplasmic reticulum (ER) of the major *Drosophila* rhodopsin Rh1 in the R1-6 photoreceptor cells (Schneuwly et al., 1989; Shieh et al., 1989; Colley et al., 1991; Stamnes et al., 1991, reviewed by Ranganathan et al., 1991; Smith et al., 1991). Unlike most cyclophilins, ninaA is an integral membrane protein, the membrane anchoring being provided by the C-terminal extension (Stamnes et al., 1991). It seems likely that the extensions in CYP-4 and Cyp4p will serve a similar function.

***cyp-5***

*cyp-5* was identified by McCombie et al. (1992) as part of the *C. elegans* cDNA sequence project. We have not yet determined the physical position of this gene. As with *cyp-3*, the *cyp-5* cDNAs appear to be full-length, encoding a 189 amino-acid protein (Figure 6). However, we cannot exclude the possibility that the analyzed cDNAs do not contain the whole coding region as there is no

in-frame stop codon upstream of the first methionine. The CYP-5 protein is most similar to the CypB (S-CyP) class of cyclophilins. CypB is a secreted form of cyclophilin (Caroni et al., 1991; Hasel et al., 1991; Price et al., 1991; Spik et al., 1991) found in the ER and other calcium storage compartments (Arber et al., 1992).

### *C. elegans* calcineurin

What signal transduction pathway might calcineurin be involved in *C. elegans*? To address this question, we have cloned a *C. elegans* calcineurin A gene using degenerate PCR primers. The worm gene maps to chromosome *IV*, close to *mec-3* (Figure 1), and is highly homologous to its mammalian and yeast counterparts (Figure 7).

## Discussion

We have reported in this paper the existence of five *C. elegans* cyclophilins. Alignment of the *C. elegans* CyPs with other cyclophilins currently available in the databases shows that the worm cyclophilins are highly homologous to the other members of the family (Figure 8).

Why does *C. elegans* have so many different cyclophilins? One possibility is that the worm has found it useful to have several genes expressing proteins with the same or similar function (possibly to provide redundancy or increased regulatory flexibility). For example, CYP-1, CYP-2, and CYP-3 are more similar (72-80% identical) to each other than to any other cyclophilin (Table 1), suggesting that they might have arisen from a relatively recent duplication event. Furthermore, these three proteins, but not CYP-4 or CYP-5, contain a unusual 7

residue insertion in the first half of the sequence. Interestingly, the only other cyclophilins in which this 7-residue insertion can be found have been isolated from higher plants (Figure 8). The high degree of conservation in this stretch between plants and nematodes suggests that it is functionally important. What such a function might be, and why this insert has not been found in other species remains to be determined. However, it should be mentioned that other proteins also have variations in the length of this region, with certain proteins having less (CYP-4 and the prokaryotic CyPs) and other more (*S. cerevisiae* Cyp4p, mammalian NK-TR).

Distinct cyclophilins subfamilies have also been identified in other species. The different genes appear to encode for proteins with distinct subcellular localizations. For example, CypA is the major cytosolic cyclophilin, while CypB is a common secreted (ER) form and a third form is imported into mitochondria (reviewed in Trandinh et al., 1992). One possibility is that these proteins function as general chaperonins in their respective subcellular compartments, and are otherwise interchangeable. This hypothesis is supported by the observation that in *Neurospora crassa* the same protein is used in both cytosol and mitochondria, the precursor to the mitochondrial form being synthesized with an additional signal sequence as a result of alternative splicing (Tropschug et al., 1988). Other cyclophilins however have more restricted expression patterns and roles: *Drosophila ninaA* for example appears to be required only for the proper folding of the Rh1 rhodopsin in the R1-R6 photoreceptor cells (Schneuwly et al., 1989; Shieh et al., 1989; Colley et al., 1991; Stamnes et al., 1991.

The high degree of similarity between CypA and *C. elegans* CYP-1, CYP-2, and CYP-3 could suggest that all three proteins might be worm CypA

equivalents. However, CYP-1 is more likely to be directed to an organelle, as it contains what appears to be a signal peptide at its N-terminus. The amphipatic nature and positive charge of this signal peptide suggests that it could direct import into mitochondria; thus, CYP-1 could well be the mitochondria cyclophilin.

CYP-5 appears to be the most abundant cyclophilin in *C. elegans*. Five independent *cyp-5* cDNA clones were isolated in the course of the *C. elegans* cDNA sequence project (out of about 2,000 to 3,000 independent and randomly selected cDNAs), compared to two *cyp-3* cDNAs and no cDNAs at all for *cyp-1*, *cyp-2*, and *cyp-4*. Based on its sequence, we propose that CYP-5 is the *C. elegans* CypB (secretory) cyclophilin. Two major activities in the *C. elegans* life cycle – the generation of a new cuticle during the larval molts and the production of yolk in adult hermaphrodites – involve the secretory pathway. CYP-5 might be required for either or both of these processes.

The fifth *C. elegans* cyclophilin, CYP-4, is a divergent member of the family. We do not know what its function might be, although the similarities between CYP-4 and *Drosophila* ninaA suggest that CYP-4 is an integral membrane protein, and might, like ninaA, have a more stringent substrate specificity.

We have also cloned by PCR a *C. elegans* homolog of the large (A) subunit of the protein phosphatase calcineurin, the target of the CyP-CsA complex. Identification of the expression pattern of this gene *in C. elegans* will help to determine what role this phosphatase plays in the worm. Both mammals and yeast have 2 calcineurin genes. Our degenerate PCR experiments have identified only one homolog in *C. elegans*. However, it quite possible that a

second calcineurin A gene exists in *C. elegans* and that we simply were unable to amplify it with the primers used. This question will need to be resolved (possibly through low stringency Southern blots on worm genomic DNA), as the presence of two calcineurin genes might complicate the search for mutations in this gene due to the possibility of functional redundancy.

## Experimental Procedures

### Degenerate PCR

Degenerate primers (CCIAARACIGCIGARAAYTTYMGIGCIYT and CCRAAIACIACITGYTTICCRTCIARCCA) from regions conserved between vertebrate and yeast cyclophilins were used to amplify *C. elegans* cyclophilin homologs from genomic DNA. Two distinct bands were observed on agarose gels. The PCR products corresponding to these bands were subcloned into pBluescript II (Stratagene, La Jolla, CA), and the sequence of the insert determined.

For calcineurin, two degenerate primers (ATGTGYGAYYTNYTNTGG and IGGIGRCCARTANGGRTGNGG) from regions conserved between human and yeast calcineurin A were used to amplify *C. elegans* calcineurin from genomic DNA. Unusual bases: I, inosine (a synthetic base that can pair with all four natural bases); R, A or G; Y, C or T; M, A or C; N, A, C, G, or T.

### Physical mapping

Cloned gene fragments were placed onto the physical map by hybridization to a set of ordered YACs covering most of the *C. elegans* genome dot blotted onto a single nylon membrane (known as the YAC polytene filter).

### Molecular Biology

Standard molecular biology protocols (Sambrook et al., 1989) were followed except where noted. All plasmid subcloning was done into pBluescript II (Stratagene, La Jolla, CA). The DNA sequence of fragments of interest was

obtained by dideoxy sequencing using T7 polymerase (Sequenase, USB), following the protocol suggested by the manufacturer.

### *cyp* cDNAs

The cloned *cyp-1* and *cyp-2* PCR fragments were used to screen a *C. elegans* lambdaZAP cDNA library. From approximately 500,000 plaques, we isolated 2 independent *cyp-1* and 3 independent *cyp-2* cDNAs. The sequences present at the ends of the inserts were determined for all clones, and the sequence of a representative cDNA was determined (one strand only) for each gene.

### Sequence analysis

Sequences were analyzed using the GeneWorks program (IntelliGenetics, Mountain View , CA). Protein sequences were aligned using the GAP program from the GCG package (Genetics computer Group, Madison, WI); multi-protein alignments were subsequently optimized by hand.


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**Table 1: Degree of conservation between various cyclophilins.**

Percent identity and similarity (in parentheses) between *C. elegans* CYP-1, CYP-2 (partial sequence), CYP-3, CYP-4, CYP-5, *Drosophila melanogaster* Cyp-1 (Stamnes et al., 1991), ninaA (Schneuwly et al., 1989; Shieh et al., 1989), *S. cerevisiae* Cyp1p (Haendler et al., 1989), Cyp2p (Koser et al., 1991), Cyp3p (McLaughlin et al., 1992), Cyp4p (Franco et al., 1991), Mouse (*M. musculus*) CypA (Hasel and Sutcliffe, 1990), CypB (Hasel et al., 1991), CypC (Friedman and Weissman, 1991), and human (*H. sapiens*) CypA (Haendler et al., 1987). Protein sequences were compared using the GAP program from the GCG package (Genetics computer Group, Madison, WI).

**Percent Identity (Percent Similarity)**

| Organism: | *C. elegans* | | | | | *D. melanogaster* | | *S. cerevisiae* | | | | *M. musculus* | | | *H. sapiens* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein: | CYP-1 | CYP-2 | CYP-3 | CYP-4 | CYP-5 | Cyp-1 | ninaA | Cyp1p | Cyp2p | Cyp3p | Cyp4p | CypA | CypB | CypC | CypA |
| *C. elegans* CYP-1 | – | | | | | | | | | | | | | | |
| *C. elegans* CYP-2 | 75 (79) | – | | | | | | | | | | | | | |
| *C. elegans* CYP-3 | 72 (81) | 82 (90) | – | | | | | | | | | | | | |
| *C. elegans* CYP-4 | 36 (56) | 39 (59) | 40 (59) | – | | | | | | | | | | | |
| *C. elegans* CYP-5 | 56 (71) | 57 (71) | 60 (78) | 36 (55) | – | | | | | | | | | | |
| *D. melanogaster* Cyp-1 | 70 (81) | 70 (83) | 73 (84) | 39 (58) | 59 (73) | – | | | | | | | | | |
| *D. melanogaster* ninaA | 43 (64) | 52 (71) | 45 (70) | 31 (56) | 44 (66) | 42 (65) | – | | | | | | | | |
| *S. cerevisiae* Cyp1p | 65 (77) | 66 (80) | 66 (80) | 45 (61) | 59 (77) | 70 (81) | 47 (68) | – | | | | | | | |
| *S. cerevisiae* Cyp2p | 51 (65) | 54 (65) | 57 (71) | 36 (62) | 52 (70) | 55 (70) | 45 (68) | 56 (72) | – | | | | | | |
| *S. cerevisiae* Cyp3p | 61 (71) | 66 (73) | 66 (76) | 40 (58) | 53 (71) | 64 (75) | 42 (63) | 70 (81) | 49 (64) | – | | | | | |
| *S. cerevisiae* Cyp4p | 34 (51) | 33 (56) | 39 (60) | 24 (47) | 33 (53) | 36 (53) | 32 (58) | 36 (56) | 36 (58) | 41 (65) | – | | | | |
| *M. musculus* CypA | 71 (80) | 70 (81) | 70 (80) | 40 (59) | 58 (73) | 74 (84) | 44 (66) | 64 (77) | 52 (69) | 61 (73) | 34 (54) | – | | | |
| *M. musculus* CypB | 59 (74) | 65 (78) | 64 (81) | 40 (63) | 70 (81) | 61 (78) | 48 (67) | 61 (76) | 53 (68) | 53 (71) | 34 (59) | 64 (78) | – | | |
| *M. musculus* CypC | 55 (67) | 59 (73) | 58 (75) | 36 (61) | 63 (70) | 58 (74) | 46 (65) | 58 (73) | 53 (69) | 53 (70) | 34 (59) | 58 (72) | 65 (77) | – | |
| *H. sapiens* CypA | 71 (81) | 71 (81) | 70 (80) | 39 (59) | 58 (74) | 74 (83) | 43 (65) | 65 (77) | 52 (69) | 64 (74) | 33 (54) | 96 (98) | 64 (79) | 57 (73) | – |

**Figure 1: Map position of *C. elegans* cyclophilins and calcineurin**

*C. elegans* cyclophilins and calcineurin A were placed onto the genetic map based on their physical position. Clones were mapped to the physical map by hybridization to the YAC polytene filters (see text). The positions of *cyp-3* and *cyp-5* have not been determined.

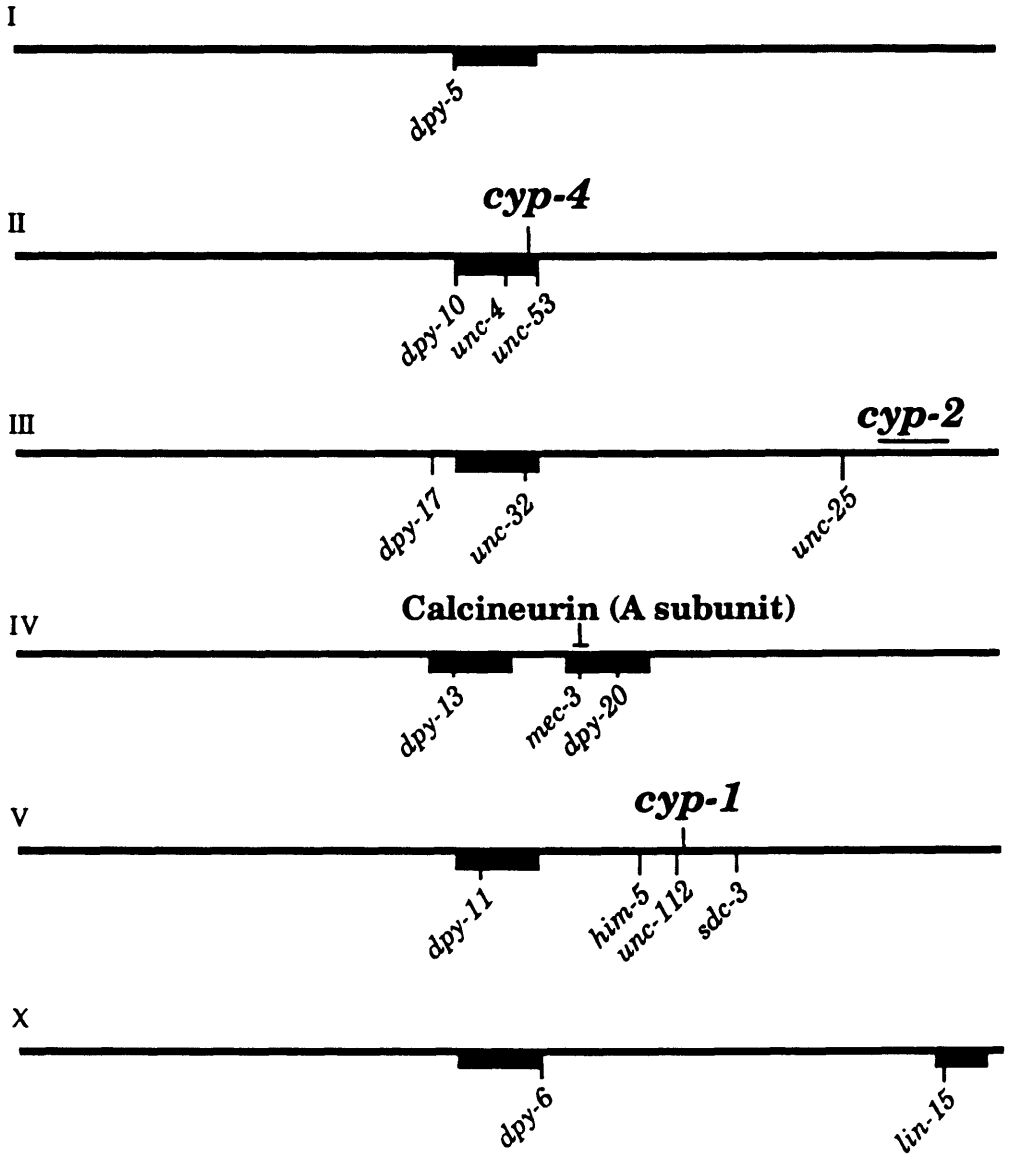
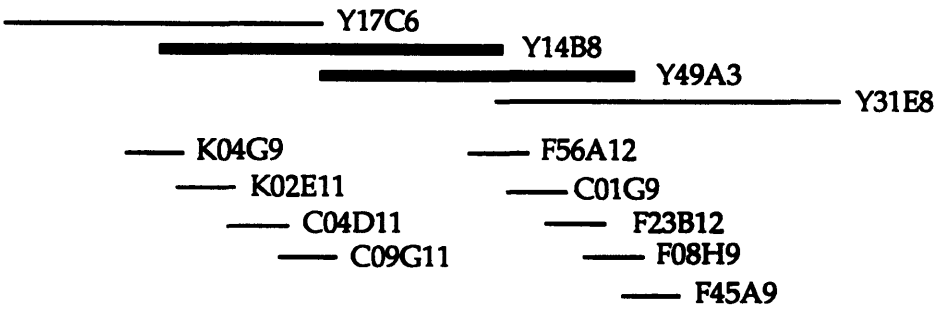

I
dpy-5
cyp-4
II
dpy-10
unc-4
unc-53
III
cyp-2
dpy-17
unc-32
unc-25
Calcineurin (A subunit)
IV
dpy-13
mec-3
dpy-20
cyp-1
V
dpy-11
him-5
unc-112
sdc-3
X
dpy-6
lin-15

**Figure 2: *cyp-1***

a) Physical map. *cyp-1* locates to a region on the right arm of chromosome *V* that is covered only by YACs. Clones that hybridize to the *cyp-1* probe are shown in bold. The gap in the cosmid coverage to which *cyp-1* maps is approximately 100 kb wide. Only YACs and cosmids tested for hybridization to *cyp-1* are shown.

b) *cyp-1* cDNA and protein sequences. The predicted CYP-1 protein sequence is shown in single letter code below the cDNA sequence. The first nine bp of the cDNA (underlined) are identical to the SL1 splice leader sequence, suggesting that *cyp-1* transcripts are *trans*-spliced to SL1. ∇, position of a 63 bp intron found in the genomic sequence (this intron is between residues 278 and 279 in the cDNA sequence).

**a**




**b**

```
     1        10        20        30        40        50
     .        .         .         .         .         .
  1  AAGTTTGAGGTAAAATGAAATTTCTACTCCGTGCCTCCTCACTTGCCGGT   50
                   M  K  F  L  L  R  A  S  S  L  A  G

 51  CAATCACTTCGATTCGCCTCTCAAAGACCGAAGTGGTTTTTCGACGTGAG  100
     Q  S  L  R  F  A  S  Q  R  P  K  W  F  F  D  V  S

101  TATTGGAGAAGAACCAGCAGGACGTGTCACCATGGAGTTGTTCAACGATG  150
      I  G  E  E  P  A  G  R  V  T  M  E  L  F  N  D  V

151  TTGTCCCAAAAACAGCAGAGAATTTCCGCGCATTGTGCACCGGTGAGAAG  200
       V  P  K  T  A  E  N  F  R  A  L  C  T  G  E  K

201  GGCGTCGGAGAGCAAGGAGTTGCACTTCACTTCAAGGGATCAAAATTCCA  250
     G  V  G  E  Q  G  V  A  L  H  F  K  G  S  K  F  H
                                  ▽
251  CAGAATCATTCCAGAGTTCATGATTCAGGGAGGAGATTTCACTCGCCACA  300
      R  I  I  P  E  F  M  I  Q  G  G  D  F  T  R  H  N

301  ACGGAACTGGTGGTGAATCAATCTACGGAAATAAATTCAAGGACGAGAAT  350
       G  T  G  G  E  S  I  Y  G  N  K  F  K  D  E  N

351  TTCGATCTTAAGCACACCGGACCAGGATGCCTTTCAATGGCCAACGCTGG  400
     F  D  L  K  H  T  G  P  G  C  L  S  M  A  N  A  G

401  ACCAAACACCAACGGATCTCAGTTTTTCATTTGCACAGTCGACACTCCAT  450
      P  N  T  N  G  S  Q  F  F  I  C  T  V  D  T  P  W

451  GGCTTGATGGAGGTCACGTCGTTTTCGGACAAGTCACCGACGGCATGTCT  500
       L  D  G  G  H  V  V  F  G  Q  V  T  D  G  M  S

501  GTTGTGAAGAAGATCGAAAAGATGGGATCCCGATCCGGAGCCCCAGCCAA  550
     V  V  K  K  I  E  K  M  G  S  R  S  G  A  P  A  K

551  GACTGTCACAATCGCCGATTGCGGAGAGTTGAAGAGCGAGTAACTTCATT  600
      T  V  T  I  A  D  C  G  E  L  K  S  E  *

601  GTTTTTTTTGTTTCAAAAATATTTTTCATTAGCGTTAGCAAATTGTTATT  650
651  ATTTTTTAATTAATTGTTTTTAATTAGTAAAGTTTTAGTTTTTAAAAAAA  700
701  AA                                                  702
```

**Figure 3: *cyp-2***

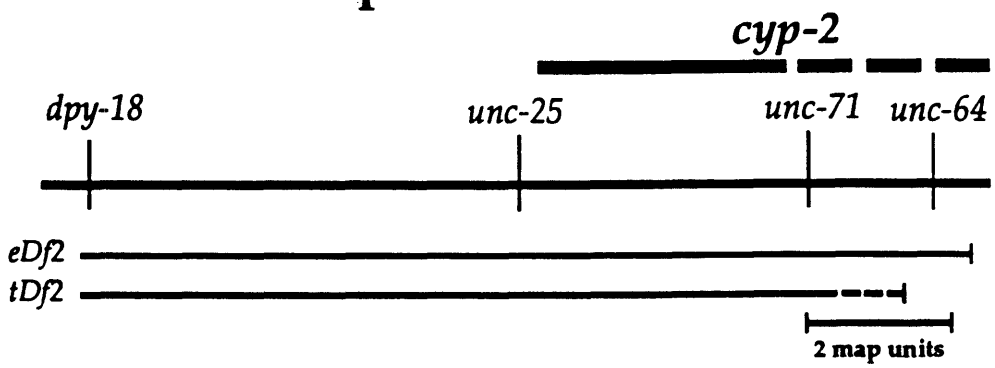
a) Genetic map of the *cyp-2* region. *cyp-2* maps to the right of *unc-25* on the right arm of chromosome *III*. There are no cloned genes to the right of *unc-25*, thus making the position of *cyp-2* very vague.

b) Physical map. Clones that hybridize to the *cyp-2* probe are shown in bold. Only YACs and cosmids tested for hybridization to *cyp-1* are shown.

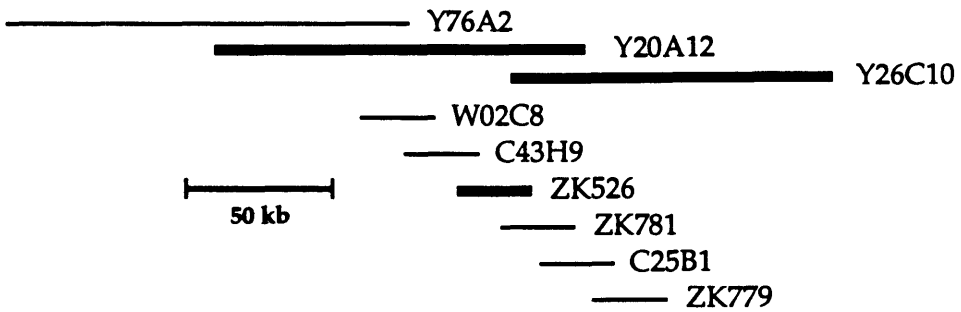
c) Restriction map and intron/exon structure of the *cyp-2* locus. The intron/exon structure shown is based on comparison of the *cyp-2* genomic and cDNA sequences. Open boxes: coding sequences (CDS); horizontal lines: untranslated regions; arrow: direction of transcription. The 5' end of the transcript is missing.

d) *cyp-2* cDNA and protein sequences. The predicted CYP-2 protein sequence is shown in single letter code below the cDNA sequence. ∇, position of a 157 bp intron found in the genomic sequence (this intron is between residues 214 and 215 in the cDNA sequence).

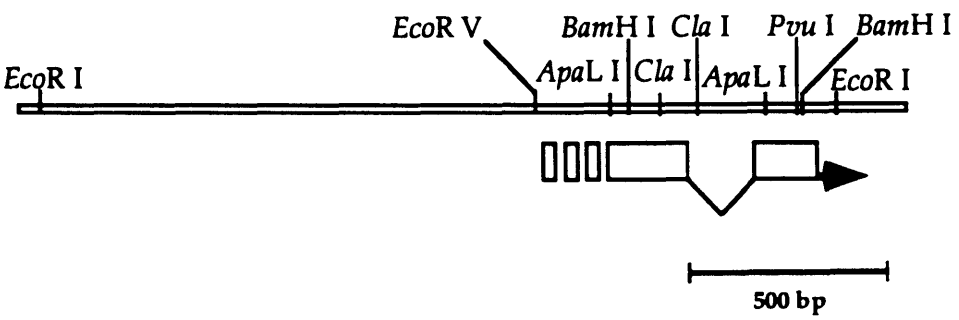
# a Genetic map




# b Physical map




# c Restriction map



# d

```
    1        10        20        30        40        50
    .        .         .         .         .         .
 1  CTTCCGCGCTCTGTGCACCGGCGAGAAAGGAAAAGGGAAATCGGGCAAGA   50
      F  R  A  L  C  T  G  E  K  G  K  G  K  S  G  K  K
51  AGTTGCACTTTAAAGGATCCAAATTTCATCGCATCATCCCCGAGTTTATG  100
        L  H  F  K  G  S  K  F  H  R  I  I  P  E  F  M
101 ATCCAGGGCGGCGATTTTACGGAGGGAAACGGCACCGGCGAATCGATTCA 150
      I  Q  G  G  D  F  T  E  G  N  G  T  G  E  S  I  H
151 TGGCGAGAAGTTTGACGACGAGAACTTCAAGGAGAAGCATACCGGACCCG 200
        G  E  K  F  D  D  E  N  F  K  E  K  H  T  G  P  G
201 GAGTCTTGTCGATGGCAAACTGTGGGGCGAACACAAACGGCTCGCAATTC 250
          V  L  S  M  A  N  C  G  A  N  T  N  G  S  Q  F
251 TTCCTGTGCACAGTCAAAACCACGTGGCTAGACGGGAAACACGTGGTTTT 300
      F  L  C  T  V  K  T  T  W  L  D  G  K  H  V  V  F
301 CGGCAAAGTGATCGAAGGAATGGATGTGGTGAAGGCGATCGAGTCGAAAG 350
        G  K  V  I  E  G  M  D  V  V  K  A  I  E  S  K  G
351 GATCCGAAGATGGGGCACCGTCGGCTCCGTGCGTCATTGCTGACTGCGGA 400
          S  E  D  G  A  P  S  A  P  C  V  I  A  D  C  G
401 GAAATGAAGTGATGCTTCTCGAAGAACAACACTGGAGAATTCGTCTTTTT 450
      E  M  K  *
451 ATTGGATAAATATGATTTTTAAAAAAAAAAAAAAAAAAA             490
```

**Figure 4: *cyp-3***

*cyp-3* cDNA and deduced protein (single letter code) sequence.

```
      1         10         20         30         40         50
      .         .          .          .          .          .
  1   GCNAGTNTNGAGTAATTTCCCTATTCTTGCCTCAACAATGAGCCGCTCAA    50
                                                M  S  R  S  K

 51   AGGTCTTTTTCGACATCACCATCGGTGGTAAGGCTTCCGGACGTATCGTC   100
        V  F  F  D  I  T  I  G  G  K  A  S  G  R  I  V

101   ATGGAGCTTTACGATGACGTTGTCCCAAAGACCGCCGGTAATTTCCGCGC   150
      M  E  L  Y  D  D  V  V  P  K  T  A  G  N  F  R  A

151   TCTCTGCACCGGAGAAAATGGAATCGGAAAGTCTGGAAAGCCACTCCACT   200
        L  C  T  G  E  N  G  I  G  K  S  G  K  P  L  H  F

201   TCAAGGGATCCAAGTTTCATCGCATCATCCCGAACTTCATGATCCAGGGA   250
        K  G  S  K  F  H  R  I  I  P  N  F  M  I  Q  G

251   GGAGACTTCACCCGCGGAAACGGAACTGGAGGAGAATCCATCTACGGAGA   300
      G  D  F  T  R  G  N  G  T  G  G  E  S  I  Y  G  E

301   GAAGTTCCCAGACGAGAACTTCAAGGAGAAGCACACCGGACCAGGAGTCC   350
        K  F  P  D  E  N  F  K  E  K  H  T  G  P  G  V  L

351   TCTCCATGGCTAATGCTGGACCAAACACCAACGGATCTCAATTCTTCCTC   400
        S  M  A  N  A  G  P  N  T  N  G  S  Q  F  F  L

401   TGCACCGTCAAGACCGAGTGGCTCGACGGAAAGCACGTCGTTTTCGGACG   450
      C  T  V  K  T  E  W  L  D  G  K  H  V  V  F  G  R

451   TGTCGTCGAAGGATTGGACGTCGTGAAGGCTGTCGAATCCAACGGATCCC   500
        V  V  E  G  L  D  V  V  K  A  V  E  S  N  G  S  Q

501   AATCCGGAAAGCCAGTCAAGGACTGCATGATCGCTGACTGTGGACAACTC   550
        S  G  K  P  V  K  D  C  M  I  A  D  C  G  Q  L

551   AAGGCATAAATTAATTGCACCCGAAATCTCCTTCATCTCTTGATTATATA   600
      K  A  *

601   TTTTCAGTATTCAACTAATCCATATTTTTCTCATAAACCTCAGTTATCCC   650
651   CG                                                  652
```

**Figure 5: *cyp-4***

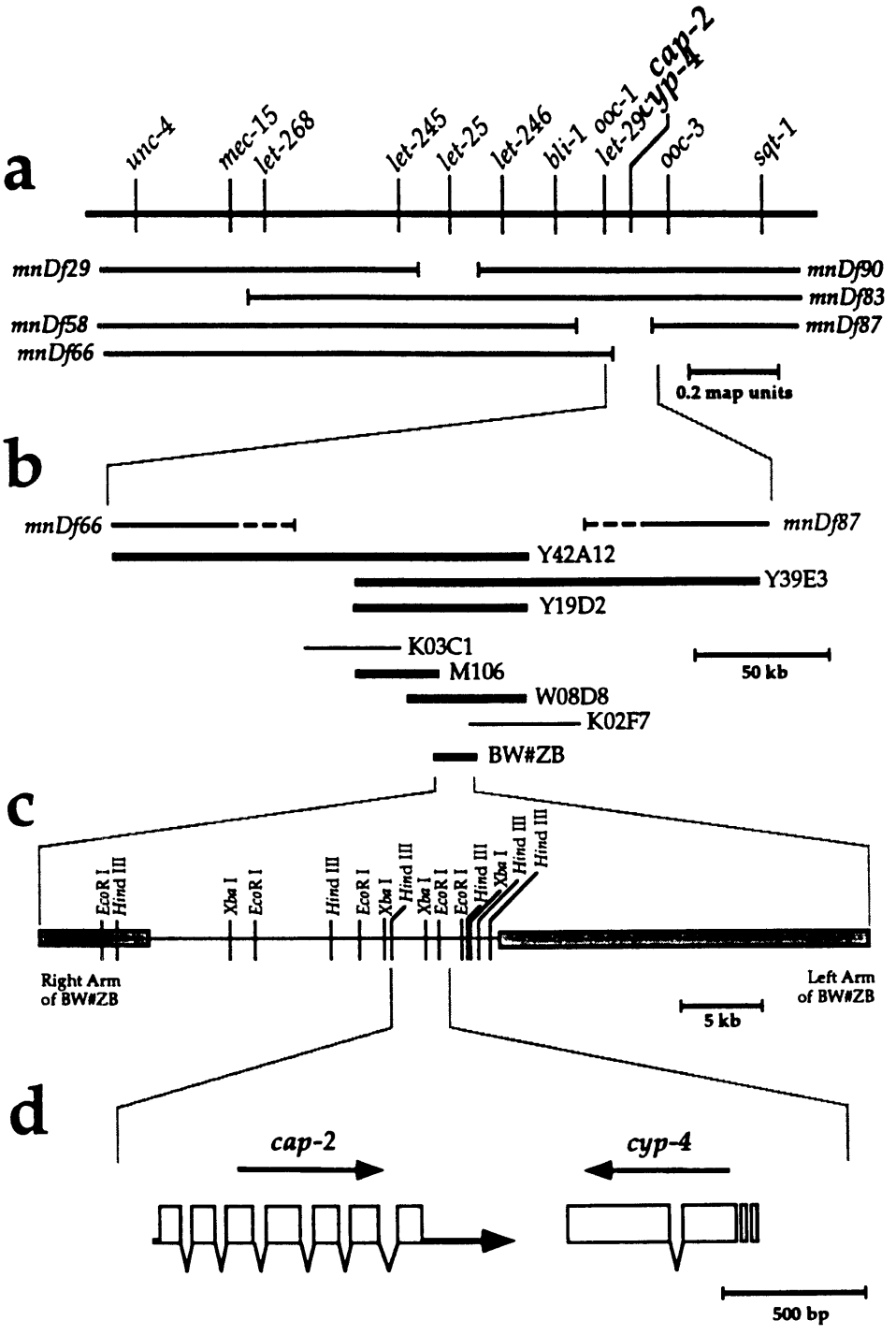
a) Genetic map of the *cyp-4* region. Neighboring genes and deficiencies used to localize *cyp-4* more precisely on the genetic map are shown.

b) Physical map. Clones containing *cyp-4* are shown in bold. The maximum extent of the deficiencies *mnDf66* and *mnDf87*, which do not take out *cyp-4*, are indicated.

c) Restriction map of phage BW#ZB, which contains both *cyp-4* and *cap-2*.

d) *cyp-4* and *cap-2* transcripts. Open boxes: coding sequences (CDS); horizontal lines: untranslated regions; arrow: direction of transcription. As no *cyp-4* cDNA has been isolated, the extent of the 3' UTR is unknown. Similarly, the 5' end of the *cyp-4* CDS has not been determined.

e) *cyp-4* genomic and protein sequences. Exonic sequences are shown in bold, intronic sequences in small letters. The deduced amino acid sequence is shown in single letter code below the DNA sequence. The *cap-2* transcripts extends up to residue 956 (underlined sequence).


a
unc-4
mec-15
let-268
let-245
let-25
let-246
bli-1
ooc-1
let-29
cap-2
cyp-4
ooc-3
sqt-1
mnDf29
mnDf90
mnDf83
mnDf58
mnDf87
mnDf66
0.2 map units
b
mnDf66
mnDf87
Y42A12
Y39E3
Y19D2
K03C1
M106
W08D8
K02F7
BW#ZB
50 kb
c
EcoR I
Hind III
Xba I
Right Arm of BW#ZB
Left Arm of BW#ZB
5 kb
d
cap-2
cyp-4
500 bp

# e

```
1         10         20         30         40         50
.         .          .          .          .          .
  1 TCCACGTAATGGCTCCAGTTACAAGCAATAAAGCAGCTGTTTTGGATAAT   50
          M  A  P  V  T  S  N  K  A  A  V  L  D  N
 51 GATACAGTTCGGTACAGCCGAGTTAAAGAAAAAATGCTTTTGTGCGGCTT  100
    D  T  V  R  Y  S  R  V  K  E  K  M  L  L  C  G  L
101 GTTACAAATTTTGGGCCATTTGAATCTGGAATTATTCGCGCCGAAAGTTC  150
     L  Q  I  L  G  H  L  N  L  E  L  F  A  P  K  V  P
151 CGAAGGCCTGTGAAAACTTCATCACACATTGTTCGAATGGATATTATAAT  200
       K  A  C  E  N  F  I  T  H  C  S  N  G  Y  Y  N
201 AACACCAAATTCAATCGGTTAATTAAAAACTTTATGgtaaatttattata  250
    N  T  K  F  N  R  L  I  K  N  F  M
251 aaaatagttaaagttataacaaaaaataattaaaaatttcagCTGCAAGG  300
                                           L  Q  G
301 AGGCGACCCAACTGGTACAGGACATGGTGGAGAATCAATTTGGGATAAGC  350
     G  D  P  T  G  T  G  H  G  G  E  S  I  W  D  K  P
351 CATTCTCAGATGAATTCATCTCCGGTTTCTCCCATGATGCTCGAGGTGTG  400
       F  S  D  E  F  I  S  G  F  S  H  D  A  R  G  V
401 CTCTCGATGGCTAATAAGGGCTCTAACACCAACGGATCACAATTCTTCAT  450
    L  S  M  A  N  K  G  S  N  T  N  G  S  Q  F  F  I
451 TACATTCCGGCCGTGCAAGTATTTGGACAGAAAGCACACAATTTTTGGGA  500
     T  F  R  P  C  K  Y  L  D  R  K  H  T  I  F  G  R
501 GACTTGTTGGAGGACAGGATACACTAACGACGATTGAAAAGTTGGAAACT  550
       L  V  G  G  Q  D  T  L  T  T  I  E  K  L  E  T
551 GAAGAGGGAACTGATGTTCCGATGGTTTCAGTTGTTATTATGAGAGCTGA  600
    E  E  G  T  D  V  P  M  V  S  V  V  I  M  R  A  E
601 AGTATTTGTGGATCCATTTGAAGAAGCTGAGAAAGAAGTTCAAGCTGAGA  650
     V  F  V  D  P  F  E  E  A  E  K  E  V  Q  A  E  R
651 GAGCAGAGATTTTGAAGAAAAGTTTCTTAATTCTATTACTAATTTTAAAA  700
       A  E  I  L  K  K  S  F  L  I  L  L  L  I  L  K
701 CTAATGATATCTATTTTCAGCTTCTAAGGACGCAGCTTCATTAGCAAATA  750
    L  M  I  S  I  F  S  F  *
751 AAAAAGCTAAAGAAACTGCAACAAAACCAGAAGCAGTTGGAACTGGAGTC  800
801 GGCAAGTACATGAAGTCGGCCGCAGCTGTGAATAAACGACAAGGAAAAAT  850
851 GGAAGATGTACCCTTGGAGGCCGCAAAGAAAACTAAATTTGCAAGAGCAG  900
901 GCCTTGGAGATTTCTCAAAGTGGTGATGAAGACCCTCTCTTTTTTTTTAA  950
951 ATTTAAGGAATAAAATACTTTCATCGAAAATATAATTGAAATGAAAAGTA 1000
```

**Figure 6*: cyp-5***

*cyp-5* cDNA and deduced protein (single letter code) sequence. X, exact nature of the residue unknown due to uncertainties in the DNA sequence.

```
    1        10        20        30        40        50
    .        .         .         .         .         .
  1 CTTNTTGTGGCGGACGTGCTCGCTGTCGGAGCTCATGCTCAGCGGAGACG   50
                                          M  L  S  G  D  D

 51 ATGCCAAAGGACCAAAGGTCACTNACAAGGTCTACTTTGACATGGAAATT  100
      A  K  G  P  K  V  T  X  K  V  Y  F  D  M  E  I

101 GGAGGAAAACCAATCGGACGTATCGTCATCGGACTTTTCGGAAAAACCGT  150
    G  G  K  P  I  G  R  I  V  I  G  L  F  G  K  T  V

151 CCCAAAGACCGCCACCAACTTCATTGAGCTCGCCAAGAAGCCAAAGGGAG  200
     P  K  T  A  T  N  F  I  E  L  A  K  K  P  K  G  E

201 AAGGATATCCAGGAAGCAAGTTCCACCGAGTCATCGCAGACTTCATGATT  250
      G  Y  P  G  S  K  F  H  R  V  I  A  D  F  M  I

251 CAAGGAGGAGATTTCACCCGTGGAGATGGAACCGGAGGACGCTCTATCTA  300
    Q  G  G  D  F  T  R  G  D  G  T  G  G  R  S  I  Y

301 CGGAGAGAAGTTCGCCGACGAGAACTTCAAGCTGAAGCACTACGGAGCCG  350
     G  E  K  F  A  D  E  N  F  K  L  K  H  Y  G  A  G

351 GATGGTTGTCGATGGCCAACGCTGGAGCTGATACCAATGGATCCCAATTC  400
      W  L  S  M  A  N  A  G  A  D  T  N  G  S  Q  F

401 TTCATCACCACAGTCAAGACCCCATGGCTCGATGGACGCCACGTCGTTTT  450
    F  I  T  T  V  K  T  P  W  L  D  G  R  H  V  V  F

451 YGGAAAGATCCTCGAAGGAATGGATGTGGTCCGCAAGWTCGAACAGACCG  500
     G  K  I  L  E  G  M  D  V  V  R  K  X  E  Q  T  E

501 AGAAGCTTCCTGGGGACCGCCCGAAGCAAGACGTCATCATCGCCGCTTCT  550
      K  L  P  G  D  R  P  K  Q  D  V  I  I  A  A  S

551 GGACACATCGCCGTCGACACTCCATTCTCTGTTGAACGCGAGGCCGTCGT  600
    G  H  I  A  V  D  T  P  F  S  V  E  R  E  A  V  V

601 CTAAGCTCAAATGACTTCTCTTTTTAAATATCGATCTGTGATTCTCCTGA  650
     *

651 CTTCAGACACCTGAATTAAAGAGTAATTCGAGTTTCTTTTTAATTTATCG  700
701 ACTTTTTTGTGCGTTTCCAATTAAATAATATTACAAAGT              739
```

**Figure 7: calcineurin**

Alignment of the translated *C. elegans* calcineurin PCR fragment with the two human (Guerini and Klee, 1989) and yeast (Cyert et al., 1991) calcineurins. Positions and sizes of three introns found in the *C. elegans* DNA sequence are indicated.

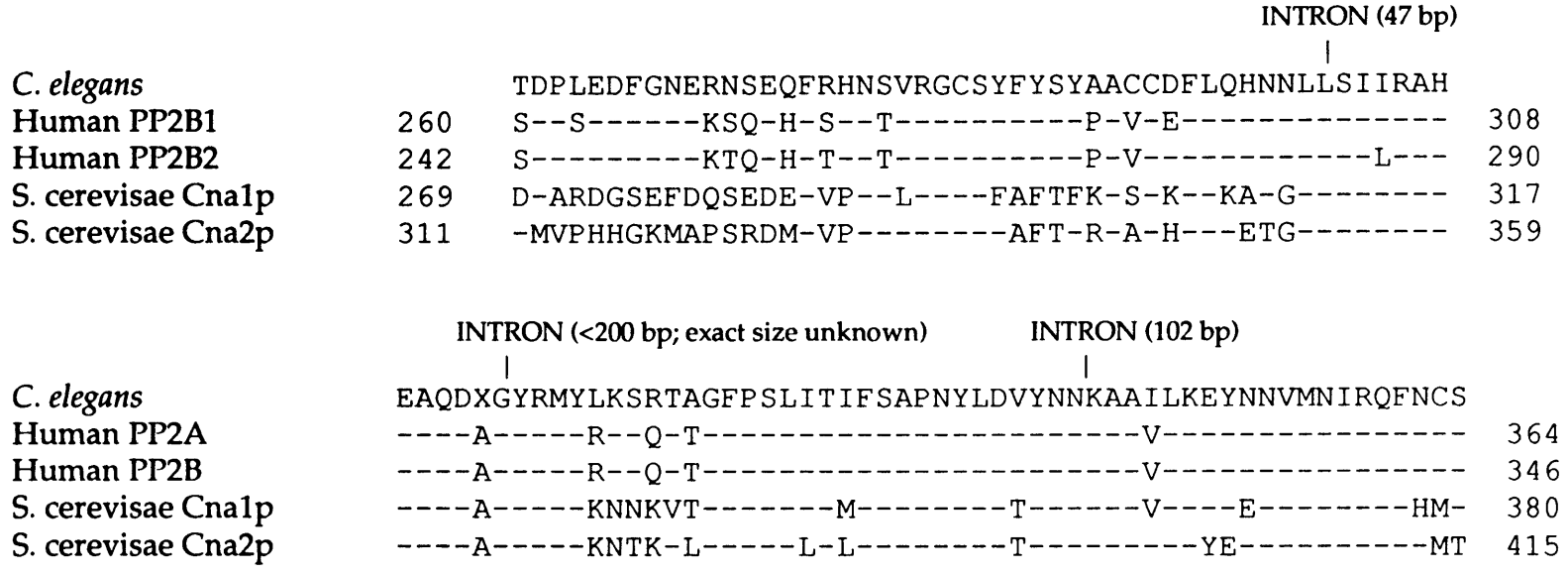
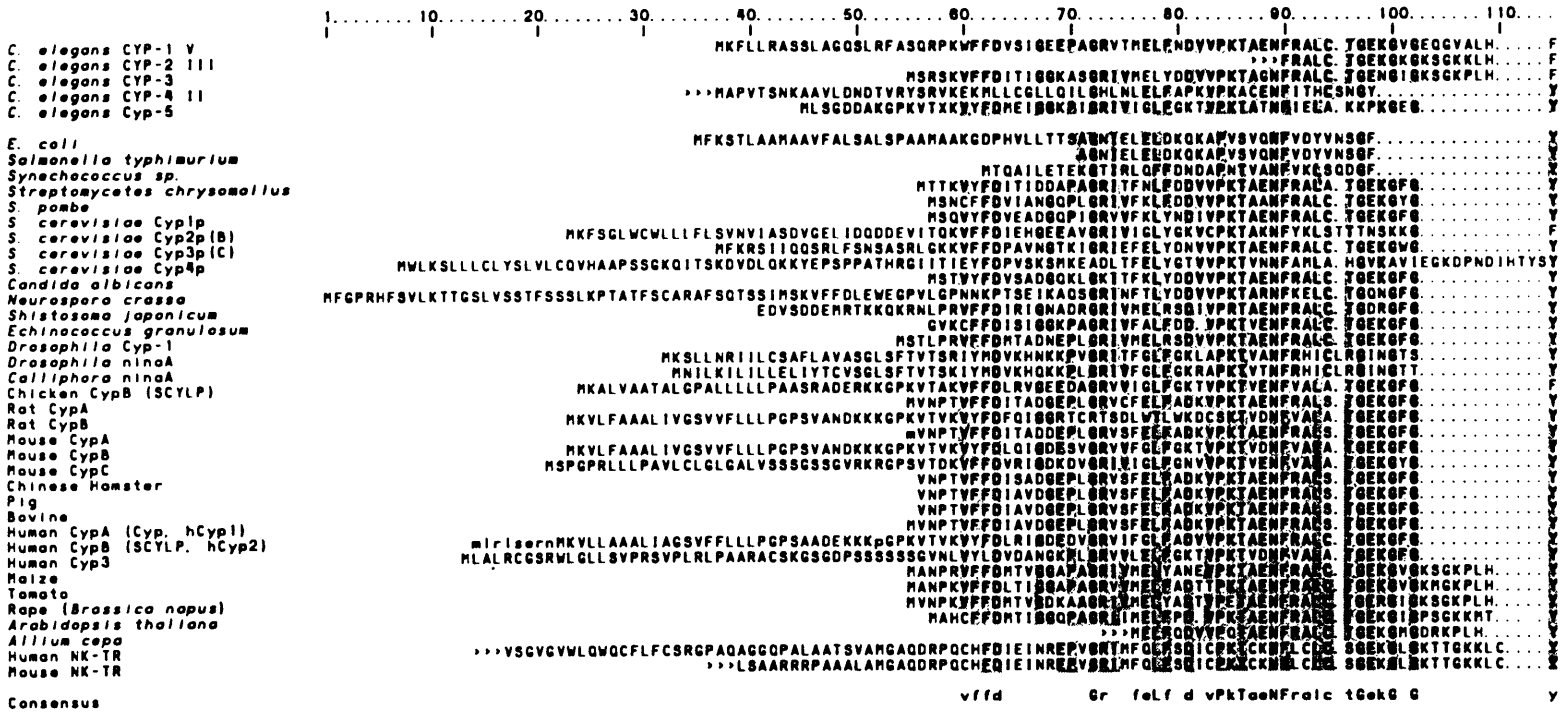
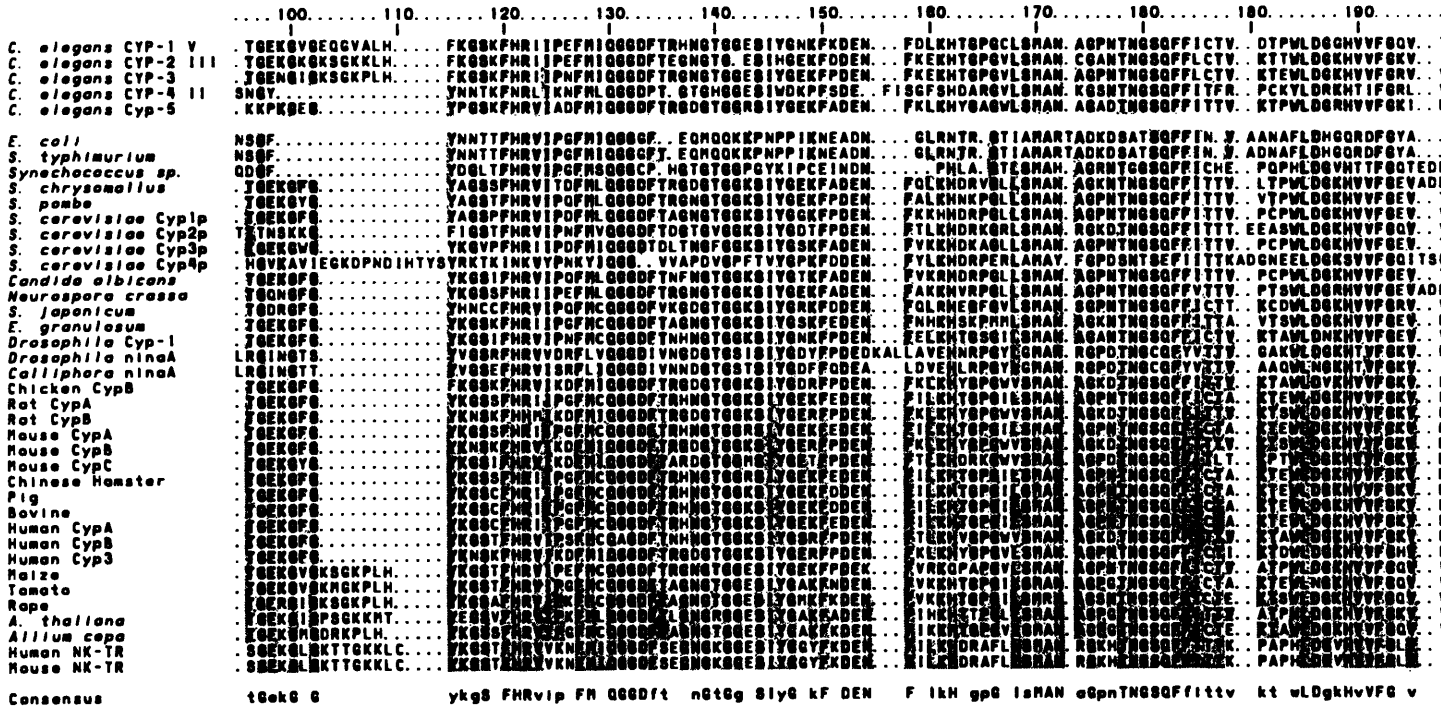

INTRON (47 bp)
C. elegans TDPLEDFGNERNSEQFRHNSVRGCSYFYSYAACCDFLQHNNLLSIIRAH
Human PP2B1 260 S--S------KSQ-H-S--T----------P-V-E-------------- 308
Human PP2B2 242 S---------KTQ-H-T--T----------P-V------------L--- 290
S. cerevisae Cna1p 269 D-ARDGSEFDQSEDE-VP--L----FAFTFK-S-K--KA-G-------- 317
S. cerevisae Cna2p 311 -MVPHHGKMAPSRDM-VP--------AFT-R-A-H---ETG-------- 359
INTRON (<200 bp; exact size unknown)
INTRON (102 bp)
C. elegans EAQDXGYRMYLKSRTAGFPSLITIFSAPNYLDVYNNKAAILKEYNNVMNIRQFNCS
Human PP2A ----A-----R--Q-T----------V---------- 364
Human PP2B ----A-----R--Q-T----------V---------- 346
S. cerevisae Cna1p ----A-----KNNKVT-------M--------T------V----E--------HM- 380
S. cerevisae Cna2p ----A-----KNTK-L-----L-L--------T---------YE----------MT 415

**Figure 8: Conservation between *C. elegans*, prokaryotic, vertebrate, fungal, and plant cyclophilins.**

Alignment of the five *C. elegans* cyclophilins discussed in this paper with cyclophilins from *E. coli* (Liu and Walsh, 1990), *Salmonella typhymurium* (Tran et al., 1990), *Synechococcus sp.* (A. Kaplan, unpublished, Genbank Acc# P29820), *Streptomycetes chrysomallus* (Pahl et al., 1992), *S. pombe* (de Martin and Philipson, 1990), *S. cerevisiae* Cyp1p (Haendler et al., 1989), Cyp2p (Koser et al., 1991), Cyp3p (McLaughlin et al., 1992), Cyp4p (Franco et al., 1991), *Candida albicans* (Koser et al., 1990), *Neurospora crassa* (Tropschug et al., 1988), *Shistosoma japonicum* (Argaet and Mitchell, 1992), *Echinococcus granulosum* (Lightowlers et al., 1989), *Drosophila melanogaster* Cyp1 (Stamnes et al., 1991), ninaA (Schneuwly et al., 1989; Shieh et al., 1989), *Calliphora vicina* ninaA (Ondek et al., 1992), Chicken CypB (SCYLP) (Caroni et al., 1991), Rat CypA , CypB (Iwai and Inagami, 1990; Arber et al., 1992), Mouse (*Mus musculus*) CypA (Hasel and Sutcliffe, 1990), CypB (Hasel et al., 1991), CypC (Friedman and Weissman, 1991), Chinese hamster CypA (Bergsma and Sylvester, 1990), Pig (Fisher et al., 1989; Takahashi et al., 1989), Bovine CypA (Harding et al., 1986), Human CypA (Haendler et al., 1987), CypB (Price et al., 1991), Cyp3 (Bergsma et al., 1991), Maize (Gasser et al., 1990), Tomato (Gasser et al., 1990), Rape (Gasser et al., 1990), *Arabidopsis thaliana* (Bartling et al., 1992), *Allium cepa* (S. A. Clark, unpublished, Genbank Acc# L13365), human NK-TR (Anderson et al., 1993), and mouse NK-TR (Anderson et al., 1993). Consensus line: small letters, residue found in ≥ 50% of all sequences; capital letters, residue found in ≥ 90% of all sequences. Residues in the individual sequences that are identical to the consensus line are shaded. >>>, rest of sequence not shown, because it is either not available (*C. elegans* sequences), or without homology to cyclophilins (other sequences).


1 10 20 30 40 50 60 70 80 90 100 110
C. elegans CYP-1 V
C. elegans CYP-2 III
C. elegans CYP-3
C. elegans CYP-4 II
C. elegans Cyp-5
E. coli
Salmonella typhimurium
Synechococcus sp.
Streptomycetes chrysomallus
S. pombe
S cerevisiae Cyp1p
S. cerevisiae Cyp2p(B)
S cerevisiae Cyp3p(C)
S. cerevisiae Cyp4p
Candida albicans
Neurospora crassa
Shistosoma japonicum
Echinococcus granulosum
Drosophila Cyp-1
Drosophila ninaA
Calliphora ninaA
Chicken CypB (SCYLP)
Rat CypA
Rat CypB
Mouse CypA
Mouse CypB
Mouse CypC
Chinese Hamster
Pig
Bovine
Human CypA (Cyp, hCyp1)
Human CypB (SCYLP, hCyp2)
Human Cyp3
Maize
Tomato
Rape (Brassica napus)
Arabidopsis thaliana
Allium cepa
Human NK-TR
Mouse NK-TR
Consensus


C. elegans CYP-1 V
C. elegans CYP-2 III
C. elegans CYP-3
C. elegans CYP-4 II
C. elegans Cyp-5
E. coli
S. typhimurium
Synechococcus sp.
S. chrysomallus
S. pombe
S. cerevisiae Cyp1p
S. cerevisiae Cyp2p
S. cerevisiae Cyp3p
S. cerevisiae Cyp4p
Candida albicans
Neurospora crassa
S. japonicum
E. granulosum
Drosophila Cyp-1
Drosophila ninaA
Calliphora ninaA
Chicken CypB
Rat CypA
Rat CypB
Mouse CypA
Mouse CypB
Mouse CypC
Chinese Hamster
Pig
Bovine
Human CypA
Human CypB
Human Cyp3
Maize
Tomato
Rape
A. thaliana
Allium cepa
Human NK-TR
Mouse NK-TR
Consensus

# CURRICULUM VITAE


| | |
|---|---|
| Name: | Asa Abeliovich |
| Address: | 1039 Massachusetts Ave., Apt. 10B<br>Cambridge, MA 02138 |
| Telephone: | home (617) 547-6902<br>work (617) 253-6522 |
| Birth Date: | May 17, 1964 |
| Birth Place: | Afula, Israel |
| Nationality: | U.S. |
| Social Security No. : | 353-60-9237 |


Secondary School Education:

Urbana High School, Urbana, IL. 9/78 - 6/82.

Undergraduate Education:

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. 9/82 - 6/86, B.S.

Department of Humanities, Massachusetts Institue of Technology, Cambridge, MA. 9/82 - 6/86, B.S.

Graduate Education:

Harvard University-Massachusetts Institute of Technology Division of Health Sciences and Technology, Cambridge, MA. 9/86 - present, medical student.

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. 9/88 - present, graduate student.

Research Positions:

Research Assistant, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. 9/87- 9/88, laboratory of Dr. Susumu Tonegawa.

Research Assistant, Children's Hospital, Harvard Medical School, Boston, MA. 2/87 - 9/87, laboratory of Dr. Stuart Orkin.

Research Assistant, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. 2/84 - 6/86, laboratory of Dr. Irving London.

Scholarships:

Medical Scholar Training Program, 9/88 - present.

Publications:

A. Abeliovich, D. Gerber, O. Tanaka, M. Katsuki, A. M. Graybiel, S. Tonegawa. (1992). On Somatic Recombination in the Central Nervous System of Transgenic Mice. Science 257, 404-408.

Ishikawa, H., Li, Y., Abeliovich, A., Yamamoto, S., Kaufman, S. H. E., and Tonegawa, S. (1993). Cytotoxic and interferon γ-producing activities of γδ T cells in the mouse intestinal epithelium are strain dependent. Proc. Natl. Acad. Sci. USA 90, 8204-8208.

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